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Development of an improved diagnostic test for sheep scab based on biomarkers

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Declaration

I declare that the work reported in this thesis is solely my own work, unless where otherwise indicated, and has not been submitted for any other degree or professional qualification.

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Beth Wells.

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Abstract

Sheep scab is a highly contagious ectoparasitic disease caused by the mite, *Psoroptes ovis*, which causes intensely pruritic lesions with severe dermatitis and is a major welfare and production issue in the UK national flock. In an attempt to improve disease control, sheep scab was recently made notifiable in Scotland indicating that early diagnostic tests will be crucial to the success of this legislation. A sensitive and specific antibody based assay has been developed which can diagnose early infestation, but which does not indicate current disease status post-treatment due to residual circulating antibody levels. However, studies into host biology and response to disease have illustrated the potential use of biomarkers (BMs) in diagnostics as indicators of disease progression and the effectiveness of treatment regimes, including a recent microarray analysis which identified over 600 host genes differentially expressed in circulating leukocytes following *P. ovis* infestation. As many of these genes encoded proteins known to be involved in inflammatory responses, this data was used in the search for potential BMs.

Initially the genes were filtered and ranked, using bioinformatic analysis, to identify the most promising BM candidates and then evaluated using Western blot analysis against a range of sera from *P. ovis* infested and naïve sheep. Promising results were obtained for a complement binding protein, C4BPB, showing it was rapidly up-regulated following infestation and correlated with disease progression as determined by lesion size development. The ovine *C4BPB* gene was successfully sequenced for the first time and a recombinant form of this protein expressed in *E. coli.* Antibodies, raised in rabbits against ovine rC4BPB, were used to develop a sandwich ELISA, results from which suggested the potential of C4BPB as a BM for sheep scab as it indicated current disease status post-infestation and post-treatment.

The major ruminant acute phase proteins (APPs) serum amyloid A (SAA) and haptoglobin (Hp) were then investigated using commercially available assays, as previous studies indicated they were effective markers of inflammatory disease in ruminants. Results from these analyses indicated that both APPs responded positively to infestation with *P. ovis* but this was not statistically significant until 4 weeks post-infestation. After treatment, the APPs declined rapidly, as described by their short half life of less than 3 days following successful treatment, compared with 56 days for the estimated half life of the host antibody against the mite antigen Pso o 2. Further statistical analysis of the APP response suggested that SAA was the more discriminatory marker, with lower pre-infestation levels and higher sensitivity at the estimated optimum cut-off values. The possibility of using a signature of BMs, as an alternative to a single BM, was discussed as a method of increasing the sensitivity and specificity of the improved test, along with the potential of combining the BM diagnostic with the existing antibody assay. It was concluded that this would provide a highly sensitive and specific test for sheep scab which would diagnose early infestation as well as indicating current disease status post-treatment, providing a highly beneficial tool to the sheep industry to aid the control of this disease.

Abbreviations

bp	base pairs
ºC	degrees centigrade
cDNA	complementary deoxynucleic acid
cm	centimetre
DAB	3, 3'diaminobenzidine
dH_2O	distilled water
dNTP	2'- deoxynucleotide - 5'– triphosphate
DNA	deoxynucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
g	grams
g	gravitational force
hr	hour
HRP	horse radish peroxidase
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl-1-thio-β-D-galactosidase
kDa	kilo Dalton
kg	kilogram
L	litre
LB broth	Luria-Bertani broth
M	molar
MES	2- (n-morpholino) ethanesulfonic acid
mg	milligram
ml	millilitre
mM	millimolar
mm	millimetre
mRNA	messenger ribonucleic acid

MW	molecular weight
ng	nanogram
µg	microgram
µl	microlitre
µM	micromolar
OD	optical density
OP	organophosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	$-\log_{10}$ (hydrogen ion concentration)
p.i	post-infestation
p.t	post-treatment
qPCR	real time PCR
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
spp.	species
TAE	tris-acetate acid-EDTA buffer
tris	tris (hydroxymethyl) amino methane
Triton X-100	octylphenoxypolyethoxethanol
UV	ultraviolet
V	volt
vs	versus
W	watt
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Chapter 1: General introduction

1.1 Disease

Sheep scab, or Psoroptic mange, is an ectoparasitic disease and one of the most contagious diseases of sheep in the UK (Kirkwood, 1986). Although known as a disease which affected the health and welfare of sheep as far back as 180 BC, the mite responsible, *Psoroptes ovis*, was not identified until 1809 (Hering, 1838). Sheep scab seriously affects the welfare of sheep (Sargison et al., 1995) but also has economic implications for the livestock industry and, as such, costs the UK sheep industry in excess of £8 million per annum, mainly in treatment costs (VLA, 2010). The overall impact of sheep scab on a flock is wide ranging and if factors such as increased ewe and lamb mortality, increased time to finishing, loss in body condition, secondary infections and reduced fleece value are included along with treatment costs, sheep scab infestation has the potential to turn a profit of £5.27 per ewe into an overall loss of £13.57 per ewe on a typical lowland sheep farm (Stubbings, 2007).

The disease has been widespread in the world's sheep producing countries, with varying degrees of success at eradication and control. For example it was eradicated successfully from Norway in 1894, New Zealand in 1885, Canada in 1929 and Sweden in 1934 but in South American countries, such as Brazil and Argentina, the disease has continued to spread, partly because of an increase in the incidence of acaricide resistance problems (Kirkwood 1986). In the UK, during the first half of the 20th Century, a stringent programme of compulsory dipping with the organochlorine lindane, along with movement restrictions resulted in the eradication of sheep scab in 1952 (Kirkwood, 1986). However, sheep scab was reintroduced in 1973 through the importation of infested sheep from Ireland to a farm in Lancashire

and for the next 19 years unsuccessful attempts were made to eradicate it by compulsory dipping twice per annum until 1988 and annual dipping from 1988 until 1992 (French et al., 1999). Over this 19 year period, only 1480 outbreaks of scab in UK flocks were reported and, with growing concerns about the effect of acaricides on the environment and human health, deregulation of the control of sheep scab occurred in 1992 when compulsory national treatment and control programmes were removed (French et al., 1999). Since then the incidence of the disease has increased to such a level that it is now considered endemic in the UK national flock (Van den Broek and Huntley, 2003a) with the national annual prevalence estimated at 7000 outbreaks in 2004 (Bisdorff et al., 2006). This resulted in calls for a national scab eradication programme which was led by the National Sheep Association (NSA) and the steering group The Sustainable Control of Parasites in Sheep (SCOPS) (NSA, 2010). These programmes aimed to progress from a nationwide awareness campaign through to targeted eradication programmes, aided by the Sheep Scab Order 1997 [Ministry of Agriculture, Fisheries and Food (MAFF)], which stated that:

"It is an offence for any sheep owner to fail to treat sheep visibly affected with sheep scab along with all other sheep in the same flock, or to move sheep visibly affected with sheep scab" (MAFF, 1997).

However, policing of this act was very difficult, particularly in upland areas where an association between high sheep scab prevalence and common grazing has been observed (O'Brien, 1999, Bisdorff et al., 2006). New legislation came into force on the 17th December 2010 in Scotland which reinstated sheep scab as a notifiable disease through the Sheep Scab (Scotland) Order 2010. This current legislation

differs from previous orders by placing a legal obligation on any sheep owner to notify the Divisional Veterinary Manager if any sheep in their possession is suspected or confirmed as having sheep scab. Following positive diagnosis, all sheep on that holding must be treated and movement restrictions are applied to the farm for a minimum of 16 days following successful treatment. The order also gives provision for farmers to report a neighbour if they are suspected of having *P. ovis* infested, untreated, sheep on their premises. This is an important aspect of the legislation as it effectively brings farms where sheep have not been treated to account. It is too soon to analyse the effects of this order, but the scale of the problem is evident as during the first year of this order being in place, there were 144 notifications in total with a widespread distribution over much of Scotland (Blissitt et al., 2012).

Eradication or control of sheep scab therefore continues to provide a challenge in many sheep producing countries. In Switzerland where the disease is also notifiable, compulsory prophylactic treatment of all sheep returning to common grazing in spring resulted in the incidence of scab being reduced by more than half between 1975 and 1999, showing that progress can be made to control and reduce the incidence of sheep scab through effective management and husbandry practices (Falconi et al., 2002). Subsequently, an immunodiagnostic assay was developed (Ochs et al., 2001) resulting in the treatment of only seropositive sheep. This 'test and treat' strategy, which was applied each spring prior to the sheep being moved onto the common grazings, led to a significant reduction in seropositive flocks from 5.8% in 2001 to 2.2% in 2003, illustrating the potential of accurate diagnosis followed by treatment as an important element in control strategies (Jacober et al., 2006).

1.2 The causative mite

1.2.1 Life cycle

Psoroptes ovis belongs to the order Astigmata, family Psoroptidae, which has four genera – *Psoroptes, Chorioptes, Otodectes and Caparinia*. *Psoroptes ovis* is visible to the naked eye, appearing white on the sheep, with the adult females being approximately 0.75 mm long and slightly larger than the males. They are distinctive among the other genera of their family due to the primary genus-specific characteristic, the jointed pre-tarsus, along with the funnel shaped suckers on the end of their legs (see Figure 1.1) as opposed to the more usual bell shaped suckers (Bates, 1999a).

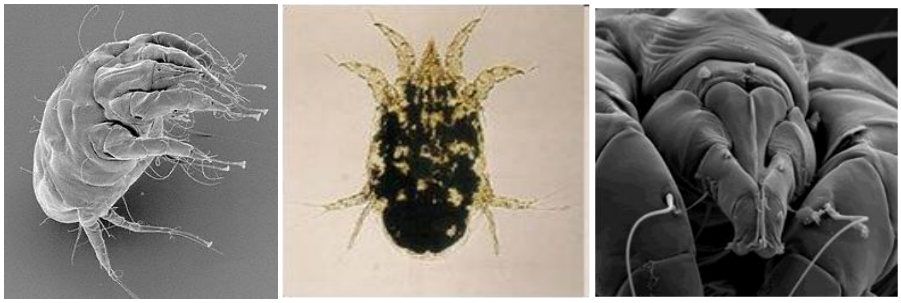

Figure 1.1: The sheep scab mite *Psoroptes ovis* showing characteristic funnel-shaped suckers and surface feeding mouthparts. Photographs reproduced with kind permission from Dr Gerry Brennan, University of Ulster and Prof. Richard Wall, University of Bristol.

Psoroptes ovis is a non burrowing mite with a five stage life-cycle progressing from egg, to larva, two nymph stages and adult as shown in Figure 1.2. All developmental stages take place on the host and under optimal conditions the life cycle can be completed in 14 days (Kirkwood, 1986). Mature females can produce up to 90 eggs

during their lifetime (Downing, 1936) and a single ovigerous female is sufficient to establish a successful infestation (Babcock and Black, 1933).

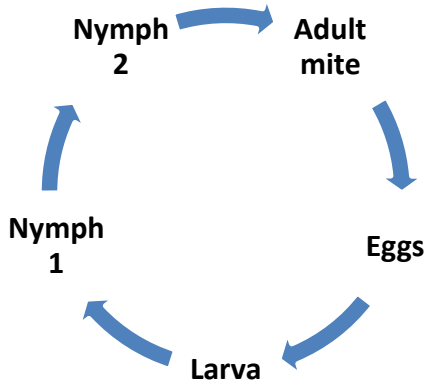

Figure 1.2: Life cycle of *P. ovis* showing the characteristic five stages all of which take place on the host.

1.2.2 Transmission

Transmission is mainly through direct contact between an infested and a susceptible animal, but can also occur indirectly as infested sheep scratch on any available object and dislodge mites (O'Brien et al., 1994). The mites, under normal conditions of temperature and humidity, can remain infective for 15-16 days off host which has implications for spreading the disease as gates, fences, bedding, transporters, handlers and shearing blades all become potential sources of infestation (O'Brien et al., 1994). Added to this is the fact that some sheep appear clinically normal but remain "carriers", with mites living in cryptic sites such as around the eyes, in the ear canals and in the folds of skin between the hind legs, where they can survive for up to 2 years (Babcock and Black, 1933). These sub-clinical carrier animals are problematic for disease control as there may be no obvious visual signs of infestation.

Infestations occur throughout the year although an increase of disease incidence has been reported in the winter months (Kirkwood, 1986), possibly due to the fleece of the sheep being thicker and longer, increasing mite survival. Management factors are also thought to be important as dipping and handling of sheep are reduced in winter months so infestations may go unnoticed (French et al, 1999).

1.2.3 Feeding

The mites live at the base of the fleece feeding on the outer layer of the skin, the stratum corneum, with mouthparts designed to abrade rather than pierce the skin (Figure 1.1) (Sinclair and Kirkwood, 1983, Van den Broek and Huntley, 2003a). Healthy sheep skin has a surface layer of lipid which provides a food source for the mites during early infestation (Sinclair and Filan, 1989). Within hours of an infestation the mites cause inflammation and serous exudation which forms an additional liquid food source (Bates, 1999b). As this occurs, the mites move out in a concentric manner onto naive skin, while the pustulated lesion (the main clinical sign of infestation) behind dries forming the crusty yellow scabs which, along with the thickening of the skin, are characteristic of sheep scab (Van den Broek et al., 2000). It has been suggested that mite proteinases may have an anticoagulant effect, enhancing serous exudation and thereby aiding mite feeding (Kenyon and Knox, 2002) and, as with other mites, the faecal pellets of *P. ovis* may be responsible for the initial inflammatory response of the host which provides the mite with its main food source (Sinclair, 1990). Extracts from mites, including *P. ovis,* contain a range of proteinases including cysteine proteinase and metalloproteinases, which are able to degrade a variety of host proteins (Nisbet and Billingsley, 1999, Nisbet and Billingsley, 2000, Kenyon and Knox, 2002). The relative activities of these

proteinases may reflect the diet of the different mite species (Hamilton et al., 2003). For example, immunoglobulin G (IgG) has been isolated from within the gut of *P. ovis*, demonstrating that host immunoglobulin (Ig) is ingested by the mite and may also be at least partially digested, as degraded components of the antibody as well as intact IgG were identified (Pettit et al., 2000). *Psoroptes cuniculi* [which may actually represent a rabbit-adapted con-specific variant of *P. ovis* (Pegler et al., 2005)] ingest erythrocytes from their rabbit host and extracts of this mite contain haemoglobin-degrading aspartyl proteinases (Nisbet and Billingsley, 1999). A study on the scabies mite *Sarcoptes scabiei* has illustrated its ability to ingest filaggrin, a structural epidermal protein, suggesting this may be a food source as well as contributing to the pathogenesis of the disease (Beckham et al., 2009).

1.3 Clinical symptoms

The clinical symptoms of sheep scab are illustrated in Figures 1.3, 1.4 and 1.5. In early infestation, they are consistent with mild pruritis which, over the course of several weeks in experimental infestations or several months in the field, progresses to an intense pruritis (Bates, 1997). Initially head shaking, lip smacking, scratching and rubbing at the infested site are the obvious behavioural abnormalities, but as the lesion continues to develop, the animal becomes increasingly restless and irritated. Biting at the flanks and scratching the body with the hind feet increases, causing shorter but more frequent bouts of grazing (Corke and Broom, 1999). The result is that the animal loses condition leading to lower live-weight gains, reduced birth-weight in lambs born to infected ewes, and reduced milk yield causing reduced weight gain in lambs (Kirkwood, 1980, Sargison et al., 1995). Wool loss from intensive scratching occurs initially over a small area which may benefit the sheep by

Figure 1.3: Farm outbreak of sheep scab showing classic disease clinical symptoms and behaviour including scratching, rubbing and wool loss. Photograph reproduced with kind permission from Prof. N. Sargison, The Royal (Dick) School of Veterinary Studies [R(D)SVS], University of Edinburgh.

Figure 1.4: Sheep scab lesion in early disease with characteristic crusty, yellow appearance. Photograph reproduced with kind permission from Dr J. Huntley, Moredun Research Institute (MRI).

exposing the mites to a less favourable micro-environment, but as the infestation and lesions spread wool loss can become severe with the remaining wool stained and wet from the serous exudate (Corke and Broom, 1999). Clearly, in winter especially, this

has consequences for heat loss and can lead to further production losses and in some cases death, particularly in hill sheep.

Figure 1.5: Severe case of sheep scab showing clinical signs of scabs, wet wool and wool loss as a result of pruritis. Photograph reproduced with kind permission from Prof. N. Sargison, R(D)SVS, University of Edinburgh.

Apart from production losses, sheep scab is also a major welfare issue as the intense pruritus quickly becomes an obsessive preoccupation for infected animals, resulting in wool loss, self trauma and open wounds, with often secondary infections developing which can lead to a decline in production and occasionally fitting and death (Kirkwood, 1980). In the majority of animals the lesions eventually resolve as a protective immune response is initiated and the animal recovers. In these cases, new wool growth removes the scabs and the new skin appears normal underneath (Bates, 1997).

1.4 Treatment and Control

The treatment options currently open to UK sheep farmers are listed in Table 1.1. The only licensed dipping products in the UK currently are organophosphates (OPs) which are effective if manufacturer's instructions are followed (SAC, 2005) as the washing action of the dip, as well as killing the mites, leads to their dislodgement and allergen removal allowing more rapid healing. However, the problems of OP dip use include harmful residues in the environment and meat, handler health issues and reported incidences of parasite resistance (Sargison et al., 2006). In addition, sheep farmers in the UK require a licence to dip and as dip disposal is under the strict regulation of the Scottish Environmental Protection Agency (SEPA), these factors add to the costs and time associated with this procedure.

Injectable endectocides, such as the macrocyclic lactones, ivermectin, doramectin and moxidectin, are a useful alternative to OP dips and many UK sheep farmers now rely on them as their sole means of treatment (Sargison et al., 2006). A study investigating the control and management of sheep scab and pediculosis in 2003-2004, found that 44% of farmers who responded (966 farmers throughout UK) treated prophylactically to prevent sheep scab, 41% by using macrocyclic lactones and 36% using OP dips, the remainder using either a combination of the above or pyrethroids. For therapeutic treatment of sheep scab 58% used macrocyclic lactones, predominantly doramectin (Bisdorff and Wall, 2008). The study concluded that due to the continued high national prevalence of sheep scab, the current control methods were not successful and that although most farmers questioned appeared to differentiate correctly between the different treatments required for lice and scab, a

large proportion of insecticide applications were taking place at the wrong time of year for maximum efficacy, i.e. June and July (Bisdorff and Wall, 2008).

Table 1.1: Treatment options and comparisons for sheep scab for UK farmers based on Scottish Agricultural College (SAC) guidelines. *Gold standard (= the optimum desired result from the treatment strategy used) and table obtained from SAC Veterinary Services (2011)

*Gold Standard	OP dips	Ivermectin	Doramectin	Moxidectin LA 2%
100% kill mites/immature	Yes	Two injections (1ml/50kg)	One injection (1ml/33kg)	One injection (1ml/20kg)
Within 24 hrs	2-3 days	Up to 7 days	Up to 7 days	Up to 7 days
Persistence>28days	Yes	No	No	Yes (60 days)
Non toxic	Concentrate toxic	Non toxic	Non toxic	Non toxic
Short meat withdrawal	70 days	42 days after 2nd injection	63 days	104 days
Convenience/equipment required	Legal controls	Need 2 injections	Single injection	Single injection
Ease of disposal	Licence required	Easy disposal	Easy disposal	Easy disposal
Cost effective 70kg ewe	70p plus disposal	20-30p x 2 doses	90p	95p

Macrocyclic lactones are extremely effective in treating sheep scab (O'Brien, 1999) and recently a long-acting moxidectin with a persistence of 60 days became

commercially available, giving farmers the option of coordinating treatment across neighbouring grazings more easily, enabling effective regional control (N. Sargison, Pers. Comm.). However, a major problem with the use of injectable endectocides is that these compounds are also used to treat internal parasites and therefore their increasing usage may increase the selection pressure for resistant gastro-intestinal nematodes (Bisdorff and Wall, 2008, Sargison et al., 2007, NSA, 2007). It is generally believed that this approach is unsustainable; therefore it was deemed imperative that alternative strategies for treatment and control were designed and implemented before sheep scab threatened the tenability of the sheep industry in the UK (Sargison et al., 2006). Since December 2010, it has been anticipated that the Sheep Scab Order (Scotland) 2010 will provide a useful legislative tool to improve long term disease control in Scotland. In addition, with heightened publicity and interest in sheep scab control, additional potential has arisen for regional control strategies, an approach which has had successful results in geographically defined areas such as the Angus glens, Cheviot hills and the Pentland hills near Edinburgh (Sargison et al., 2006). The success of these programmes has depended on cooperative treatment and control regimes within the persistence time of the acaricides used, along with rigorous biosecurity, such as quarantining and preventative treatment of newly acquired animals and thorough cleaning with an acaricide of any equipment potentially exposed to mites. Despite the problems associated with cooperative treatment in regional areas, these collaborative approaches were considered to have the potential to further control sheep scab outbreaks (Sargison et al., 2006). Currently for the rest of the UK and other countries where sheep scab is not a notifiable disease, regional control programmes could be

facilitated by using a modelling approach to identify “high risk” areas of endemic sheep scab using sheep density, height above sea level, temperature and rainfall as predictors of the probability of an outbreak (Rose et al., 2009). This approach would target high risk areas and aim to reduce scab incidence, thereby conserving the treatment compounds that remain available (Rose et al., 2009). For such enhanced control approaches to sheep scab, a resource which will be vital to any successful treatment and control campaign is the ability to accurately diagnose early, active infestation with the causative organism, *P. ovis*. Problems of misdiagnosis have been reported, in particular confusion between the symptoms produced by sheep scab mites and lice was seen as an issue (SCOPS, 2005) and as treatment for these two endoparasites is different, misdiagnosis was not only recognised as a problem for failed treatment but for selection for resistance to injectable endectocides (SCOPS, 2005). An accurate diagnostic test specific for early sheep scab infestation is therefore critical for future disease control.

1.5 Diagnosis[1]

1.5.1 Developments in ectoparasitic immunodiagnostic tools

In many cases, the diagnosis of ectoparasite infections is a more straightforward process than that for endoparasites. By definition, ectoparasites are on (or in) the skin surface and are often large and relatively immobile, e.g. ticks. There are, however, several species of ectoparasites of humans, companion and production animals which remain difficult to diagnose, because of their small size or cryptic lifestyle or because the clinical signs that they induce on the host can be confused with other infections or conditions, including, for example, those caused by *Psoroptes* and *Sarcoptes* mite species. Conventional diagnostic tests based on the direct detection of a parasite, for example, by microscopic examination of hair plucks or skin scrapings, often lack sensitivity and specificity and are usually dependent on expertise in the identification of the parasites (Swets, 1988). In addition, conventional tests may not detect subclinical infections, which can play a key role in the persistence of ectoparasites within host populations (Van den Broek and Huntley, 2003a). Clearly, improved methods of diagnosis are required and recently progress in the development of novel diagnostic methods and technologies has been achieved for ectoparasites which infest humans, companion and production animals and for which differential diagnosis is important in the selection of appropriate treatments and disease-prevention strategies (Wells et al., 2012).

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[1] Much of this section is adapted from a recently published review: WELLS, B., BURGESS, S. T., MCNEILLY, T. N., HUNTLEY, J. F. & NISBET, A. J. 2012. Recent developments in the diagnosis of ectoparasite infections and disease through a better understanding of parasite biology and host responses. *Mol Cell Probes,* 26**,** 47-53. A copy is included at the end of this thesis.

1.5.1.1 ELISA

Immunodiagnosis by enzyme linked immunosorbent assay (ELISA) has become increasingly used for key ectoparasites (Falconi et al., 2010, Ochs et al., 2001) and diagnostic tests based on this method may possess increased sensitivity and specificity compared to traditional diagnostic methods. For example, *S. scabiei* (Order Astigmata) is an obligate parasite which burrows into the skin of the host causing scabies in humans and topical (sarcoptic) mange in a variety of domestic (e.g. dogs, pigs, sheep and goats) and wild animals (e.g., foxes, raccoons, camels and wombats) (Bornstein et al., 2006). Globally, *S. scabiei* is one of the most important ectoparasites because of its ability to cause disease in the specific host or following zoonotic transmission through infestation of commercially-important as well as companion animals (Walton et al., 2004). Diagnosis has been based principally on clinical symptoms and the identification of mites from skin scrapings from affected areas (Burgess, 1994). Infestations with scabies mites can be difficult to diagnose for several reasons. Symptoms are similar to those of other dermatological conditions; parasites may not co-localise with the lesions; the mites themselves are cryptic, living in burrows and, in the case of classical scabies, only 10-20 parasites may be present on the human host (Mellanby, 1944).

An understanding of the parasite / host interaction has been used to facilitate the development of novel immunodiagnostics. The skin lesions and extreme pruritis, which are characteristic of infestations with *S. scabiei*, indicate an immediate-type hypersensitivity reaction (Falk and Bolle, 1980b). This response is inflammatory and allergic-like in nature with infiltrations of eosinophils, lymphocytes and histocytes into the areas surrounding the mite burrows (Walton and Currie, 2007). However, it

is the humoral immune response against the scabies mites that has allowed the development of immunodiagnostic tools. An adaptive immune response, involving high serum titres of immunoglobulin E (IgE) and IgG, has been demonstrated in human patients with crusted scabies (Roberts et al., 2005) and other studies confirm that scabies mite infestation leads to the production of measurable antibody in infested hosts (Falk and Bolle, 1980a). Therefore, ELISAs have been developed for the detection of mite-specific antibodies in infected goats or sheep (Falconi et al., 2010), (Rodriguez-Cadenas et al., 2010b, Rodriguez-Cadenas et al., 2010a), pigs (Jacobson et al., 1999, van der Heijden et al., 2000), dogs (Lower et al., 2001) and foxes (Bornstein et al., 2006). These assays have used native protein preparations (i.e. mite extracts) as antigens, with the attendant problems that this might bring (e.g. cross-reactivity with other species and batch-to-batch quality control issues). Other ELISAs, based on the use of recombinant proteins, or hydrophobic or hydrophilic antigen preparations have been evaluated (Hejduk et al., 2011, Kuhn et al., 2008), with variable results.

Microscopic examination of skin scrapings is generally thought to be an inefficient method of sarcoptic mange diagnosis (Hengge et al., 2006) and was recently compared with an indirect ELISA for detecting mite-specific IgG antibodies in sheep (Rodriguez-Cadenas et al., 2010a). There was a marked difference between the sensitivities of the two diagnostic tests (ELISA 87.6%; skin scraping 62.8%), but there was better agreement between the two tests in terms of their specificity (95.9% for ELISA; 100% for skin scraping), when the accuracy of the tests was compared with that of a reference method for epidemiological surveys and evaluated using the Receiver Operating Characteristic (ROC) curve approach (Rodriguez-Cadenas et al.,

2010a). The increased sensitivity of the ELISA-based test suggests that it could be used to support the diagnosis of the presence of sarcoptic mange, estimate the prevalence, indicate mange-free status, and determine the efficacy of control or treatment programs (Rodriguez-Cadenas et al., 2010a).

The diagnosis of sheep scab is also not straight forward as, although clinical symptoms of disease are evident in the advanced stages of infestation, during early infestation animals may exhibit minimal clinical signs but still represent a source of infestation (Bates, 2000). Mites from these animals are difficult to find, even when one suspects their presence, and the efficacy of the microscopic detection of mites in skin scrapings from these animals can be as low as 18% (Bates, 2009). In addition to *P. ovis*, other ectoparasites such as ticks and lice can induce skin reactions which may appear similar to those seen in early infestation with *P. ovis* (Sargison et al., 1995, Bates, 1997), and dual infestations with more than one ectoparasite are common, further confusing diagnosis. Researchers in New Zealand have developed a highly sensitive and specific assay to detect sheep louse (*Bovicola ovis*) antigen in wool fibres (Patent US 07294477, Ovita Ltd) based on the presence of an antigen in louse faeces which are ubiquitously shed as the lice move around on the skin. However, a similar test for sheep-scab, based on the detection of parasite antigen is unlikely to succeed since mites, unlike lice, do not migrate extensively across the skin surface but are generally confined to the lesion/normal skin interface or in isolated cryptic sites.

An alternative method is the detection of anti-*P. ovis* antibodies in blood; anti-mite IgG titres increase from initial detection levels at the end of the lag phase of

infestation (approximately 2 weeks post infestation) reaching peak levels at 8-12 weeks post infestation (Van den Broek et al., 2003c). Although levels of antibody can remain elevated for several months after successful treatment (Bates, 2009, Burgess et al., 2012b) serological methods have been employed successfully for the effective differential diagnosis of psoroptic mange in cattle and in sheep (Fisher, 1983, Wassall et al., 1987, Boyce et al., 1991, Lonneux et al., 1996, Ochs et al., 2001, Bates, 2000, Bates, 2009). An ELISA developed by researchers at the University of Zurich has been used both as an immunodiagnostic and an epidemiological tool to assess the seroprevalence of sheep scab in Switzerland (Falconi et al., 2002, Ochs et al., 2001). Although this assay employed a crude mixture of unspecified antigens extracted from the parasites, the test demonstrated excellent sensitivity and specificity of 93.7% and 96.5%, respectively.

These studies have thus demonstrated the potential of an immunodiagnostic assay for use in control programmes for sheep scab and encouraged the search for more specific or robust assays employing defined antigens and, in particular, recombinant proteins. The latter offer potential advantages in terms of uniformity, stability and supply of the antigen target as well as reduced production costs, which are particularly important for tests which will be produced commercially or widely distributed for use in different laboratories. Candidate antigens for such a diagnostic test have included house dust mite (HDM) allergen homologues (Huntley et al., 2004, Lee et al., 2002, Nisbet and Huntley, 2006) and while these allergens have regions which are highly conserved between mite species, suggesting important or pivotal functions in mite biology, they are sufficiently different to generate species-

specific antibodies during infestation (Lee et al., 2002, Nisbet and Huntley, 2006, Nisbet et al., 2006c). This immunogenicity suggests that they represent good candidates for the development of assays to detect specific antibodies against *P. ovis* in the host. Two such HDM allergen homologues have received particular attention, namely Pso o 1 and Pso o 2 and it is the latter that is showing particular promise as a diagnostic target. Pso o 2 is a 16kDa polypeptide with sequence homology to the group 2 allergens Der f 2 and Lep d 2 from *Dermatophagoides farinae* and *Lepidoglyphus destructor,* respectively (Pruett, 1999, Temeyer et al., 2002).

In an ELISA developed for diagnosis of sheep scab, the use of a single recombinant antigen, Pso o 2, as the diagnostic antigen reliably detected the presence of *P. ovis* on sheep within two weeks of experimental infestation (Nunn et al., 2011) and sensitivity and specificity were similar to those reported for the previous native antigen extract based assay (Falconi et al., 2002). Perhaps the most important finding from these studies was that the test could detect Pso o 2-specific antibodies in a natural outbreak before clinical signs were evident (Burgess et al., 2012b). This assay will therefore be of particular use as an aid to control this highly infectious mite by monitoring and targeting those flocks that require treatment, for example during local eradication campaigns. The use of recombinant proteins also enables the development of reliable pen-side detection methods such as lateral flow devices, and the targeted use of these in the early and rapid detection of disease will be pivotal to effective control.

The approach to further increasing diagnostic potential using molecular tools in *P. ovis* research has focused on gaining a greater understanding of the local and global

host response to infestation. In a recent study using microarray technology, the host skin response to infestation with *P. ovis* was examined and over 1500 host genes identified that were significantly differentially expressed within 24 hours post-infestation (Burgess et al., 2010). Clustering of these genes enabled their classification into temporally related groupings and network and pathway analysis of these temporal clusters identified key signaling pathways involved in the host response to infestation. This analysis resulted in the identification of a crucial role for toll-like receptor 4 (TLR4) triggering via nuclear factor-kappa B (NF-kB) to instigate the host pro-inflammatory response to *P. ovis.* Der p 2 has previously been identified as a potential trigger for TLR4, putatively acting as a functional mimic of the TLR4 accessory protein MD-2 (Trompette et al., 2009) suggesting a similar role for its homologue Pso o 2.

1.5.2 Enhancement of diagnostic tools

Even highly specific and sensitive immunodiagnostics have the limitation that they are unable to distinguish antibodies produced during active infestation from residual antibody from previous infestations when used at a single time point. For example, studies have shown that the host circulating antibody (IgG) response to *P. ovis* infestation in sheep persists for between 3-6 months following successful treatment (Bates, 1999c, Ochs et al., 2001, Burgess et al., 2012b). While this limitation may be overcome to some extent by taking repeated measurements at different time points, this may be inconvenient and provides additional costs. Studies into host biology and response to disease have illustrated the potential use of BMs as indicators of disease progression and the effectiveness of treatment regimes. Generally BMs are proteins which act as indicators of a particular biological state, e.g. active infection, and

return to normal circulating levels on disease resolution or treatment (Petersen et al., 2004). In human medicine, BMs are being studied extensively in diseases such as cancer, heart disease and Alzheimer's disease, (Arnold et al., 2011, Dubois et al., 2010, Thal et al., 2006), and are useful in early disease diagnosis, improving the accuracy of diagnosis and in the selection of therapeutics. In the veterinary field, BMs have shown potential as early indicators for a variety of infectious and/or inflammatory diseases: for example the APPs are potentially valuable diagnostic and prognostic aids in bovine respiratory disease (Heegaard et al., 2000, Godson et al., 1996) and caseous lymphadenitis (CLA) (Eckersall et al., 2007).

The potential for using BMs as an additional tool in the immunodiagnosis of ectoparasitic disease is clear; the immunodiagnostic element (e.g. an antigen-specific ELISA) can provide the specificity and sensitivity to identify the causative organism whereas elevated BM level can indicate whether the disease is active or is resolving (for example after successful treatment).

In a recent example of the use of BMs in the early detection of ectoparasite disease, the APP response was studied in Alpine ibex suffering from a natural outbreak of sarcoptic mange (Rahman et al., 2010). Serum amyloid A serum titres were increased approximately 20-fold in infested compared to control animals, with Hp serum concentrations increasing 10-fold during infestation, therefore these APPs were considered as having potential as BMs for the detection of disease. This is of particular importance in the control of sarcoptic mange in wild ibex as there is a high incidence of this condition in the wild population, and early diagnosis is likely to be critical for disease control (Rahman et al., 2010).

In relation to sheep scab, a diagnostic BM assay, especially if used in conjunction with the existing ELISA for increased specificity, would be a highly beneficial tool for the sheep industry as it may indicate current disease status post-treatment. This is likely to be particularly important in Scotland where sheep scab is notifiable and farms with sheep positively diagnosed as *P. ovis* infested are subject to movement restrictions until confirmed as successfully treated. The limitations of antibody persistence in this situation, means that the ELISA is not capable of indicating current disease status. Other situations where an integrated test including BMs may be useful include purchasing sheep from livestock markets when knowledge of the current disease status of the animals is desirable and when moving sheep between farms such as over-wintering lambs, a common practice in the Scottish upland sheep industry.

One approach to identifying BMs for the development of a diagnostic test for sheep scab, is to use data from a microarray study completed recently at MRI (Burgess et al., 2012a). Sheep scab infestation induced dramatic changes in circulating leukocytes with over 600 genes showing highly significant differential expression over the time course of infestation (Burgess et al., 2012a). More than 30 of these differentially expressed genes encoded proteins known to be secreted into the plasma during inflammatory responses in other diseases or conditions in human and animal models (Burgess et al., 2012a). In effect, these genes formed a list of potential BMs of inflammation which were also highly up-regulated in a wide range of conditions such as arthritis and psoriasis in humans, inflammatory conditions which show similarities with the underlying pathology demonstrated in the sheep scab lesion (Van den Brook and Huntley, 2003). These inflammatory proteins are highly

conserved in sequence between species which has the advantage that, at least for some of these potential BMs, there are commercial antibodies available allowing for their detection in serum and the development of a test based on their change in serum concentration during *P. ovis* infestation will represent a novel and highly sensitive assay for sheep scab.

1.6 Host Immune Response

1.6.1 Disease progression

The host immune response to *P. ovis* is a complex multi-factorial interaction where mite antigens play an important role in the initiation of the response of the host immune system (Sinclair, 1990). Infestation with *P. ovis* can be characterised into three phases, early, late and decline (Van den Broek and Huntley, 2003a) which is reflected by the phases of the host immune response. During the early phase, which happens minutes after infestation, the host skin surface becomes reddened and inflamed and, histologically, an influx of neutrophils and eosinophils can be detected along with blistering of the skin and the appearance of serous exudate on the skin surface (Van den Broek et al., 2004), illustrating a rapid host immune response to the effects of the mite through an immediate hypersensitivity type response (Van den Broek et al., 2000). In experimentally infested animals this early stage can last for 10-35 days, compared to 2-8 months in naturally occurring infestations (Bates, 1997) during which time clinical symptoms may not be evident or only apparent on close inspection (Bates, 2009). Following this pro-inflammatory phase, where mite numbers remain relatively low, the late phase begins where mite numbers increase exponentially and the lesion size expands allowing clinical diagnosis (Van den Broek

and Huntley, 2003a). The host immune response during this stage is characterised by a delayed hypersensitivity type response and production of antigen-specific IgE and to a lesser extent IgG (Van den Broek et al., 2000) which, in experimental infestations, may last for a further 2-4 weeks after which the disease moves into the decline phase as the host immune response affects mite numbers and the lesions begin to resolve (Van den Broek et al., 2004). This may happen naturally, or more commonly, in response to therapeutic intervention.

1.6.2 Innate immune response

As previously noted, the mouthparts of *P. ovis* do not appear to pierce the skin; therefore it is likely that the cause of the lesion pathology and the host's early hypersensitivity type response is due to the interaction between physical abrasion by the mite, salivary or faecal antigens and host keratinocytes, as the first point of contact (Watkins et al., 2009). The initial immune response in sheep to *P. ovis* infestation has previously been characterised as a hypersensitive inflammatory response dominated by the infiltration of eosinophils and to a lesser extent neutrophils, mast cells and lymphocytes (Rosa and Moschini, 1988, Stromberg and Fisher, 1986, Van den Broek et al., 2000). This rapid influx of eosinophils occurs during the first 24 hours at the initial site of infestation (Huntley et al., 2005, McNair et al., 2009) and it has been suggested that *P. ovis* allergens possess eosinophil chemo-attractant properties, which may lead to the increase in pro-inflammatory cytokines from epidermal cells (Van den Broek et al., 2004). This response brings about changes in the epithelium such as capillary dilation and a reduction in skin barrier function resulting in leakage on to the skin of a serous exudate (Burgess et al., 2010). Keratinocytes may also be activated by mite proteases disrupting tight

junctions between the cells, increasing permeability and producing the changes observed in the early stages of infestation (Nisbet and Billingsley, 2000; Kenyon and Knox, 2002).

The sheep scab mites are thought to use the host's innate inflammatory response for their own benefit as the exudate, resulting from the keratinocyte response to mite allergens, becomes a major food source for the mites (Huntley et al., 2005). A number of these allergens are homologues of house dust mite (HDM) allergens, and have been studied extensively (Huntley et al., 2004, Nisbet et al., 2006a). HDM allergens are contributing factors in allergic disorders of humans such as asthma and atopic dermatitis, where it is thought that allergen protease activity may enhance allergenicity (Chapman and Platts-Mills, 1987, Thomas and Smith, 1998). HDM extract which includes Der p 1, an immunodominant cysteine proteinase, increases the expression of the pro-inflammatory cytokines tumour necrosis factor-alpha (TNF) and interleukin 8 (IL8) (Lau et al., 1999). Pso o 1, a major *P. ovis* antigen, shows 64% identity at the amino acid level with Der p 1 (Nisbet et al., 2006b) and may therefore demonstrate a similar function (Lee et al., 2002).

The pro-inflammatory response to *P. ovis* infestation in sheep has recently been further characterised in a study where increased expression levels of genes transcribing the pro-inflammatory cytokines TNF; interleukin 1β (IL1β); interleukin 6 (IL6) and IL8, along with factors involved in immune cell recruitment and activation, such as intercellular adhesion molecule 1 (ICAM1), selectin E (SELE) and selectin P (SELP), were demonstrated in skin biopsies during *P. ovis* infestation (Burgess et al., 2010). *IL8* is also up-regulated in keratinocytes *in vitro* when

exposed to *P. ovis* excretory/secretory material within an hour of exposure (Watkins et al., 2009). It was proposed that, as well as being involved in neutrophil infiltration, IL8 has a role in the activation of many immune cells, including eosinophils, T cells and B cells, initiated by mites feeding on the skin (Watkins et al., 2009).

1.6.2.1 Acute phase response (APR)

The APR is at the core of the mammalian innate immune system with responses to infection, inflammation or injury that are central to bringing about the resolution of disease or trauma and promoting healing (Cray et al., 2009, Eckersall, 1995). The initiation of the APR and the resulting cascade is shown in Figure 1.6, where only the positive APPs are shown i.e. those that increase in plasma concentration in response to inflammatory disease. This inflammatory cascade leads to the release of APPs, under the influence of the pro-inflammatory cytokines IL1, IL6, IL8 and TNF.

The APPs are plasma proteins which have an important role in the resolution of infection in the early stages (Petersen et al., 2004). The high numbers of immune cells such as neutrophils, eosinophils and mast cells, which are characteristic of a sheep scab mite lesion, are thought to be the main source of pro-inflammatory cytokines (Burgess et al., 2010), which in turn activate the APR cascade resulting in increased serum concentrations of the APPs (Rahman et al., 2010). The APPs are not independent in their actions, but are linked with other innate and adaptive responses, such as the complement system, which are activated when parasites infest or invade a host.

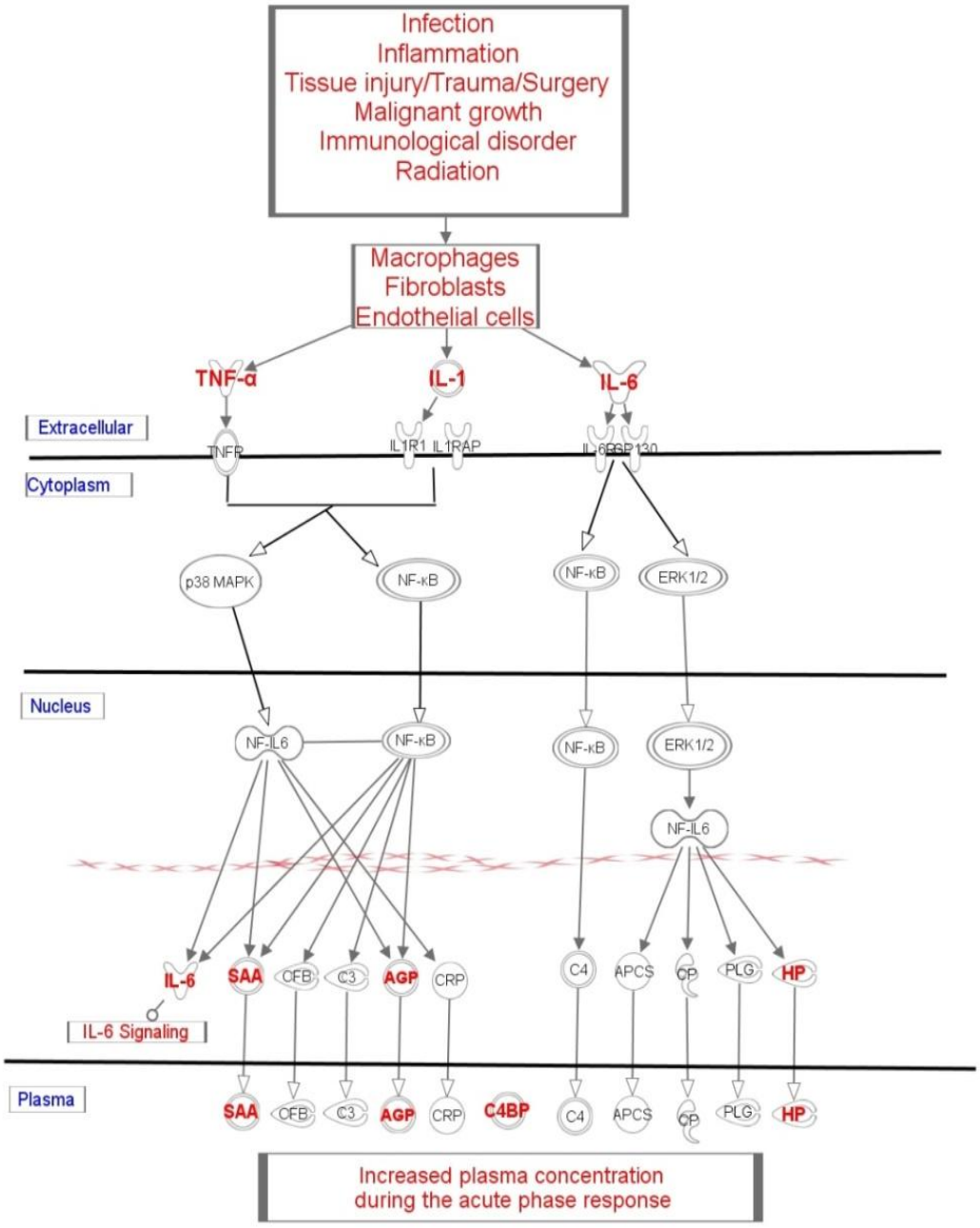

Figure 1.6: The cascade of responses of the mammalian immune system in response to disease resulting in the release of APPs and activation of the APR. The cytokines and APPs highlighted in red indicate the main pathways of the ruminant APR. Figure adapted from Ingenuity Pathway Systems (IPA). SAA = serum amyloid A; AGP = alpha-1 acid glycoprotein; C4BP = complement 4 binding protein; HP = haptoglobin.

1.6.2.2 The complement system and its interactions with mites

The complement system is part of both the innate and acquired immune systems of mammals, involving approximately 30 complement proteins and receptors, and plays a major role in the defence against invading microorganisms by inflammatory and lytic activities (Fearon and Locksley, 1996, Fearon, 1998). The complement system has principally been studied in vertebrates where it is highly conserved between species (Nonaka and Yoshizaki, 2004). It involves three cascade systems– the classical, lectin and alternative complement pathways. Activation of the complement system, when a complement component in serum binds to the surface of a pathogen, results in an enzymatic cascade ultimately leading to pathogen damage or destruction (Blom et al., 2004). The combined result of this cascade involving the 3 pathways is the tagging of pathogens with opsonins (C3b), production of inducers of inflammation (C3a, C5a) and ultimately damage to, or destruction of, the pathogen (C5b-C9) (Blom et al., 2004).

The classical pathway, although mainly activated by the acquired immune system via antibody binding, can also be activated by the APP C-reactive protein (CRP) triggered by the innate immune response and this additional binding of CRP increases the opsonisation of microorganisms (Hebecker et al., 2010). The mechanism of allergen induced activation of the complement system in humans due to HDM allergens has been described *in vitro* (Varga et al., 2003). Although IgG was required for efficient complement activation, HDM extracts activated the classical pathway by enhancing C1q binding to C1, and by binding mannan binding lectin (MBL) thereby activating the lectin binding complement pathway (Varga et al., 2003). The biologically active peptides and anaphylatoxins C3a, C4a and C5a, which

are produced in mammalian species as a result of complement activation, may contribute to the inflammatory reaction of allergic diseases (Muller-Eberhard, 1988). The HDM protease Der p 3 may be a specific activator of the complement system in humans resulting in the production of C3a, C4a and C5a, thereby contributing to the allergic and inflammatory reaction (Maruo et al., 1997) suggesting there may be potential roles for the homologous *P. ovis* antigens in sheep (S. Burgess Pers. Comm.).

1.6.3 Adaptive immune response

Acquired immunity is a specialised form of immunity involving antigen-specific lymphocytes and memory cells, thus limiting re-infection with the same organism. The adaptive immune response takes longer to develop than the innate immune response and also displays high antigen specificity, in contrast to the broad action displayed by components of the innate immune response (Coico and Sunshine, 2009).

In sheep scab, the adaptive immune response has been identified in primary infestations during the period of exponential increase in the mite population, where a rapid increase in lesion area is apparent and circulating anti-*P. ovis* specific IgE, IgM and IgG antibodies are produced (Bates, 1997, Lee et al., 2002, Van den Broek et al., 2003c). Secondary infestations were characterised by reduced lesion growth with quicker resolution (2 cm^2 peak lesion size at 1 week post infestation) compared with primary infestations (>3 cm^2 at 13 weeks post infestation) (Van den Broek et al., 2000). Mite-specific serum IgG and IgM levels in primary and secondary infestations were not significantly different (Van den Broek et al., 2003c) whereas there was a rapid, sustained, increase in *P. ovis* -specific IgE levels in sheep serum after

secondary infestations compared to those in primary infestations (Van den Broek et al., 2000). A similar response has been reported to *S. scabiei* in sheep where primary infestations were characterised by raised and sustained specific IgG levels, and a smaller but steady rise in specific IgE (Rodriguez-Cadenas et al., 2010b). In secondary infestations, the mite-specific IgE levels were significantly higher than in primary infestations and the lesion sizes and mite numbers were reduced, suggesting that development of immunity to this parasite is more closely correlated with specific IgE antibodies than to IgG (Rodriguez-Cadenas et al., 2010b) .

Many allergens, which induce the type of hypersensitive response seen in sheep scab, have enzymatic activity which typically induces a Th2 type immune response, such as those seen in chronic human inflammatory disease (Medzhitov, 2008). This response is typical of parasitic infestations, under the influence of interleukin 4 (IL4), produced by dendritic cells, which promotes the differentiation of naïve $CD4^+$ T cells into Th2 cells. The Th2 cells synthesise IL4 and interleukin 13 (IL13), which cause B cells to effect an IgE response, and interleukin 5 (IL5) which activates eosinophils, a response which has been widely researched in helminth infections (Ludwig-Portugall and Layland, 2012). It has been suggested in helminth infection that, as the host's Th2 response rarely results in the expulsion of the parasite, the worm demonstrates an immuno-modulatory capacity to ensure its survival (Moreau and Chauvin, 2010). The induction of regulatory T cells (Tregs) which occurs in the host during helminth infection is also thought to contribute to this tolerance (Everts et al., 2010). Tregs have been described as a subset of T lymphocytes which are important to immune homeostasis by controlling harmful immune responses (Sakaguchi et al., 2008). The expression of *Foxp3*, a transcription factor required for the generation of

Tregs, increases in skin cells in response to the pathogenesis involved in infestation with *P. ovis* and this process is thought to limit the tissue damage during the host immune response (McNeilly et al., 2010).

At the transcription level, our understanding of the host's response to *P. ovis* infestation has been increased by a study using microarray technology to demonstrate differential gene expression in RNA extracted from circulating leukocytes of sheep over a 6 week time course of infestation (Burgess et al., 2012a). Due to the time period studied, the results have provided information bridging the early response into established disease and have demonstrated the increased expression of genes involved in complement activation, chemokine signalling, integrin and tight junction signalling, and of *IL4* and *IL13* which are involved in the development of an allergic-type Th2 biased immune response. In addition decreased expression of those genes involved in skin barrier function and those repressed by the Th2 response was shown (Burgess et al., 2012a).

The overall picture of the immune response following mite infestation is that sheep scab mite allergens trigger the release of pro-inflammatory cytokines and activate the complement pathway, resulting in immune cell recruitment and activation. This leads to further inflammation at the infestation site and worsening of the disease symptoms, such as serous exudate which aids the establishment of the mites (Burgess et al., 2012a). Characterisation of the dominant mite allergens and improved knowledge of the host immune response at the transcription level have led to development of a immunoassay quantifying the host IgG response to a specific mite antigen Pso o 2 (Nunn et al., 2011). Although this assay can diagnose early *P. ovis*

infestation and shows high sensitivity and specificity, it cannot diagnose current disease status in recently treated sheep due to antibody persistence. However, by using microarray data generated from a study demonstrating differential gene expression in circulating leukocytes of sheep over a 6 week time course of infestation (Burgess et al., 2012a), it is anticipated that serum BMs can be identified which will improve the existing diagnostic assay by indicating current disease status post-treatment. This concept forms the basis of this study, the aims of which are described in section 1.7.

1.7 Project aims

The aims of this study are as follows:

1. The identification of potential BMs of active sheep scab infestation using data from a microarray study which analysed gene expression changes in circulating leukocytes in response to infestation with *P. ovis* (Burgess et al., 2012a). This will be achieved using bioinformatic analysis software to form a ranked list of BMs on defined criteria.
2. The characterisation and evaluation of the most promising BMs from the above list using Western blot analysis as a method for preliminary validation, followed by gene sequencing, recombinant protein expression and ELISA development for any BM where the preliminary validation showed promise.
3. Investigation into the response of the major ruminant APPs Hp and SAA to *P. ovis* infestation using existing commercially-available assays to evaluate their potential as BMs of current disease status.
4. The validation of the selected BMs with field samples from natural outbreaks of sheep scab and with samples from experimental trials, including an infestation and treatment trial designed to establish the changes in serum concentration of the selected BMs post-infestation and post-treatment.
5. Comparison of the changes in serum BM concentration after treatment to the host antibody response to the mite antigen Pso o 2 using the existing diagnostic ELISA (Nunn et al., 2011) and assessment of the possibility of integrating selected BMs with the existing antibody based diagnostic ELISA assay.

Chapter 2: Bioinformatic selection of potential biomarker candidates for diagnosis of current disease status in sheep scab infestation and their preliminary evaluation by Western blotting

2.1 Introduction

A BM can be described as a characteristic which can be objectively evaluated and validated as showing measurable changes in concentration during normal biological processes compared to those found during disease or in response to treatment (Biomarkers Definitions Working Group (2001). In practice, changes in BM concentration in response to disease or treatment must be measurable and readily detectable in biofluids, such as a secreted plasma protein. Ideally BMs should circulate at low levels in the biofluids of healthy individuals, increase in titre rapidly with a readily detectable magnitude following the onset of disease and return to pre-infestation levels soon after successful treatment or disease resolution (Atkinson et al., 2001).

In relation to sheep scab, the ultimate objective of the search for potential BMs for sheep scab was to include these BMs in an improved diagnostic test in the context of the Pso o 2 based ELISA, which does not indicate current disease status post treatment due to antibody persistence, to allow pathogen specificity.

BMs have been applied extensively in the diagnosis of many medical conditions and, increasingly, for veterinary diseases in the search for earlier diagnosis, to measure the efficacy of treatment and disease prognosis (Kulasingam et al., 2010, Liang and

Chan, 2007, Zhong et al., 2011). Recently the extent of use and range of BMs employed have both increased with advances in molecular techniques and proteomics (Pang and Argyle, 2010). BMs have had the widest application in medical oncology where they are currently used clinically for diagnostic or therapeutic purposes (Cooper et al., 2007), and in cardiovascular disease where they have become an important tool for clinicians in diagnosis, response to treatment and patient prognosis (Hochholzer et al., 2010). Recently, BM discovery has used microarray analysis in the identification of potential BMs by establishing gene expression profiles during pathogenesis. This technology has been applied to establish the occurrence of differential expression of genes in health and disease states, e.g. the identification of increased expression of histone-lysine N-methyltransferase *(EZH2)* which, in sera, indicates poor prognosis in human prostate cancer patients (Cooper et al., 2007). Microarrays are useful as a prognostic and diagnostic tool due to the unbiased approach that they allow and the high number of targets, allowing identification of signatures of genes related to disease progression, which may provide a more accurate prediction of patient recovery outcome (Cooper et al., 2007). This approach has also been used to estimate response to treatment, for example in peptide vaccination for human prostate cancer patients, by monitoring gene expression profiles in peripheral blood mononuclear cells (PBMCs) throughout treatment (Komatsu et al., 2011). Thus, in patients with advanced prostate cancer who responded to peptide-based cancer vaccinations approximately half survived longer than 900 days post-treatment whereas the remaining patients died within 300 days. BM identification, to establish patients who would most likely benefit from peptide vaccination treatment, was achieved by microarray analysis of PBMCs in

blood samples from the two patient types (survivors for >900 days c.f. those who died within 300 days) pre- and post-vaccination. Results indicated that a cluster of genes associated with abnormal granulocytes which were differentially expressed in the two patient types could be used as a BM panel to predict patient prognosis with 92% accuracy (Komatsu et al., 2011). It is anticipated that this technique may lead to improved therapeutic strategies in applying peptide vaccination to patients who would most benefit from it.

The use of microarray analysis in the understanding of the pathogenesis and underlying molecular mechanisms of livestock diseases has now also been widely applied (Jacobson et al., 2011). For example, in scrapie, data obtained from microarray analysis illustrated the differential expression of 148 genes in affected sheep compared with healthy controls, using tissue extracted from the medulla oblongata (Filali et al., 2011). Some of these differentially expressed genes, which are potential BMs of neurodegenerative disease, had previously been identified but many were novel (Filali et al., 2011) and, of the newly-identified genes, 43% were increased in concentration in tissue extracted from the medulla oblongata and 57% decreased in scrapie-affected sheep. These were characterised as immune system related genes, ion-transport, nucleotide binding or structural molecule activity genes (Filali et al., 2011). Further understanding of scrapie disease progression through transcriptional profiling, has come from a study using prescapular lymph nodes and spleen cells (Gossner et al., 2011), which confirmed the up-regulation of networks of genes associated with cell apoptosis and repression of genes involved in the inflammatory response in affected animals. Knowledge from research using

microarray technology has therefore contributed to the understanding of pathology in disease progression in scrapie which may elucidate disease progression at functional protein levels and thereby lead to the identification of potential BMs for further investigation as possible diagnostic tools. A model systems approach has also been applied to scrapie research using a murine model consisting of eight distinct mouse strain-prion strain combinations to track gene expression in the brain. This approach has provided a basis for developing models for prion replication and disease, leading to possible therapeutic approaches (Hwang et al., 2009).

Gene expression studies using microarray analysis have also been applied to sheep scab to increase understanding of the host: parasite relationship through the course of disease progression and, in particular, the mechanisms underlying the host response to the mite (Burgess et al., 2010, Burgess et al., 2012a). Many of the genes found to be differentially expressed in these studies encoded pro-inflammatory, inflammatory and anti-inflammatory proteins, including a number of complement components, e.g. complement 4 binding protein alpha (*C4BPA)* and complement 4 binding protein beta (*C4BPB*), interleukin 1 alpha and beta (*IL1A* and *IL1B*), secreted phosphoprotein (*SPP*) and the S100 calcium binding proteins (*S100A8* and *S100A9*) (Burgess et al., 2012a). Temporal clustering of gene expression data into time intervals (based on sampling 0, 1, 3 and 6 weeks post-infestation) followed by pathway analysis, resulted in a clearer picture of the signalling events which occur in circulating leukocytes following exposure to *P. ovis* mites and a better understanding of the host systemic response to infestation (Burgess et al., 2012a).

The large amount of data obtained from microarray analysis can be a useful tool for BM identification (Cooper et al., 2007), but the data requires further filtering to enable selection of the more reliable BMs for disease diagnosis. Fortunately, there is now a range of computer software available, such as Genespring GX (Agilent Technologies UK) and Ingenuity Pathway Analysis (Ingenuity Systems Inc. USA), to assist in gene selection by allowing the user to define selection criteria deemed important for the proposed function of potential BMs.

The gene expression study (Burgess et al., 2012a) used as a basis for this present study, demonstrated that circulating leukocytes could be used as "sentinels" of infection at local sites and it is hypothesised that increased expression of genes in these circulating leukocytes may initiate factors detectable in serum which were reflective of inflammation. The feasibility of evaluating these BMs therefore required the ability to detect the BM in biofluids and hence the requirements for a suitable antibody to allow a qualitative evaluation by Western blot analysis, or a quantitative one using ELISA. Further refinement of the selected BMs by bioinformatic filtering was therefore required based on the availability of commercially produced antibodies suitable for preliminary BM evaluation using Western blot analysis, as the most practical method due to the time constraints of the present study.

Western blotting is considered to be an effective technique for the validation of the specificity of an antibody, which is an essential step towards clinical translational research (Signore and Reeder, 2012). To confirm antibody specificity it is important to use a complex biological sample containing the BM, such as blood, rather than a purified protein and, when using blood, a single band at the expected MW of the BM

protein on the blot is indicative of the presence of the BM and suitability of the antibody in terms of specificity (Signore and Reeder, 2012). Western blotting is also considered a powerful technique for the immunodetection of low abundance proteins as it involves electrophoresis which separates proteins according to their MW, hence separating abundant proteins enabling visualisation of the less abundant ones and providing potential multiple immunogenic antigens for detection with specific antibodies (Kurien et al., 2011). The ability to detect low abundance proteins is further enhanced by the use of sensitive detection methods such as chemiluminescence, which depends on incubation of the Western blot with a substrate that will luminesce when exposed to the reporter conjugated to the secondary antibody. Detection is achieved by either x-ray film, or more recently by a charge coupled device (CCD) highly sensitive camera such as the ImageQuant LAS4000 system (GE Healthcare, UK). This system also allows automated semi-quantitative analysis of the bands of interest obtained using densitometry, which is useful for comparing bands within a blot, or if appropriate standards are used, it can be used to produce effective quantitative comparisons across separate blots (Alegria-Schaffer et al., 2009). The evolution of these more sensitive detection systems, which can be used in the visualisation and interpretation of Western blots, has increased the technique's usefulness in BM validation prior to clinical studies (Signore and Reeder, 2012).

The aims of this chapter were:

- To use a bioinformatic filtering approach to produce a ranked list of potential BMs for sheep scab based on the list of 621 differentially expressed genes in circulating ovine leukocytes through a time course of sheep scab infestation (Burgess et al., 2012a).

- To investigate this ranked list of potential BMs for the availability of a commercially produced antibody to further refine the list of selected proteins.

- To evaluate, by Western blot analysis, the potential BMs for which there were suitable antibodies available, to determine if they were detectable in the sera of sheep infested with *P. ovis* and if they were up-regulated across a time course of infestation. If they satisfied these criteria, the BMs would then be investigated to assess their correlation with lesion size development.

2.2 Materials and methods

2.2.1 Microarray study

The microarray data used in the filtering and selection of potential BMs were extracted from a previously published study (Burgess et al., 2012a) in which 6 sheep were infested with 50-100 live *P. ovis* mites, scab lesion sizes measured and whole blood samples obtained pre-infestation and then weekly for 6 weeks post infestation. The lesion was defined as the area of hardened, crusted scab caused by the dried serous exudate from where the mites had been feeding. The edge of the lesion was taken as the reddened edge of skin where mites were apparent. Blood samples were immediately processed to isolate circulating leukocytes and total RNA was extracted (Burgess et al., 2012a). Briefly, microarray analysis (Agilent Bovine expression microarrays, 4 x 44K format, Agilent, UK) using a single-dye (Cy3) format performed to quantify gene expression differences between circulating leukocytes pre-infestation and 1, 3 and 6 weeks post-infestation samples in all animals using the Agilent single colour expression format. Probe sets (n=21,520) were filtered to remove low quality probes (flags present or marginal) leaving 14,174 probes for statistical analysis. To identify genes differentially expressed over the time course of infestation a one way analysis of variance (ANOVA) was applied with Benjamini and Hochberg False Discovery Rate (FDR) correction at a cut-off of $p \leq 0.05$ (Genespring GX 11.0). This analysis led to the identification of 1,176 significantly differentially expressed genes over the time course. This list was further filtered using a fold change cut-off of ≥ 1.8 in at least one of the time point combinations (0 (pre-infestation) *vs* 1 week post-infestation; 0 *vs* 3; 0 *vs* 6; 1 *vs* 3; 1 *vs* 6 and 3 *vs* 6),

and provided a final list of 621 genes for the downstream analysis performed and described in this chapter.

2.2.2 Filtering and annotation of potential BMs

The 621 genes, which were differentially expressed in circulating ovine leukocytes over the time course of infestation with sheep scab (0-6 weeks), were sorted based on gene expression profile across the individual time points (0, 1, 3 and 6 week pi). The flow chart in Figure 2.1 outlines the method followed. Initially, the "biomarker filter" function within the Ingenuity Pathway Analysis (IPA) (Ingenuity Systems Inc. USA version 7.6) software package was used. Genes were considered as potential BMs if the proteins they encoded were known to be detectable in at least one of the following biofluids: blood; plasma/serum; saliva or sputum, although this information was based on IPA data for humans as there was no ovine or bovine data available. The selected genes were filtered using the "find similar entities" function within the Genespring GX software package (Agilent Technologies UK version 10.0) to assess the quality of the raw microarray data obtained for each gene based on the identification of the flags and the log2 normalised intensity data for each sample across each time point, as described below.

The raw microarray data for a gene was considered as being of sufficient quality for further analysis if the following criteria were met:

1. Quality flags for the microarray probes were assessed and accepted if they were all present ("P") except allowing for 1 absent ("A") per animal at each time point but no marginals ("M"). "P" represents a gene that was expressed to a detectable

level; “A” where it was not and “M” where it was not possible to differentiate between P and A.

If this first criterion was met, then a second was imposed:

2. The gene expression profiles (normalised log [log_2] intensity values) demonstrated an increase in gene expression at each time point for all animals (n=6).

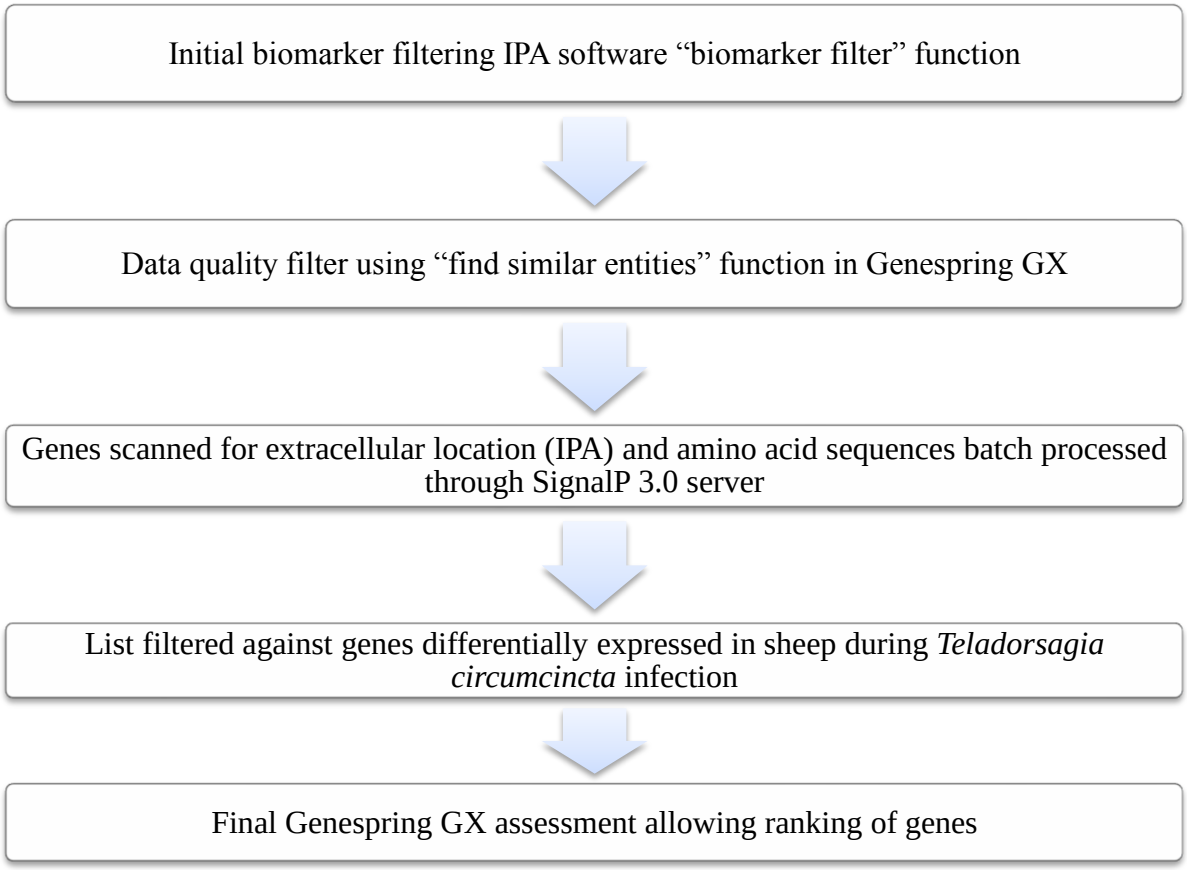

Figure 2.1: Flow diagram outlining the BM filtering process by depicting the bioinformatic functions used to filter the original list of 621 genes differentially expressed in circulating leukocytes across a time course of infestation with sheep scab to a final filtered and ranked list of 178 genes available for BM evaluation.

By individually investigating the raw expression data for all selected probes the analysis described above exceeded the stringency applied during standard approaches for microarray data analysis, which rely upon arbitrary fold-change and significance value cut-offs to limit false positive results (Dalman et al., 2012, Jeffery et al., 2006). This approach was chosen to further reduce the chances of false positive results occurring in the downstream analysis. The genes were annotated by scanning for known extracellular locations using IPA and batch processing amino acid sequences through the SignalP 3.0 server (Centre for Biological Sequence Analysis [CBS]; http://www.cbs.dtu.dk/services/SignalP) giving a prediction of the presence of a signal peptide sequence, as these two criteria indicated that the protein may be secretory and as such potentially more readily detectable in serum.

All genes were then filtered against a list of 111 genes that were found to be differentially expressed (fold change ≥1.5 and p = ≤0.05) in a parallel study in sheep, in which host responses to infection with the parasitic nematode *Teladorsagia circumcincta* were analysed (STG Burgess, Unpubl. data). This study analysed gene expression in circulating leukocytes in response to *T. circumcincta* over a 6 week time course of infection and the samples were interrogated on the same Agilent bovine microarray platform as the sheep scab infestation samples described here. *T. circumcincta* is a common gastrointestinal nematode parasite of sheep (Sargison et al., 2012); therefore removing genes differentially expressed during exposure to both parasites would increase the specificity of the list for sheep scab BMs.

The Genespring "entity inspector" function was used to re-assess the final list allowing ranking of individual genes. The rankings were derived from assessments based on three criteria:

1. The effectiveness of each gene to reflect *P. ovis* infestation over time, i.e. as the time course of infestation progressed and the gene expression was consistently increased. This criterion was graded on a scale of 1-5, (1 = excellent; 2 = very good; 3 = good; 4 = average; 5 =poor), according to the mean response curve (the curve describing the change in expression of each gene over the time course of infestation) of all animals (n = 6) over time.
2. The strength of the relationship between lesion size and the changes in gene expression through infestation. The curve obtained for the gene expression changes over the time course of infestation was examined for each individual animal and compared to the recorded lesion size at each time point over the course of infestation, thus providing an indication of the relationship between gene expression and lesion size. This criterion was graded using the same scale as in point 1 above.
3. Response magnitude (response magnitude Log2) over time was obtained from the "entity inspector" function in Genespring GX. The response magnitude refers to the overall increase in gene expression over the total time of infestation, i.e. 0-6 week p.i. The log normalised intensity value for each change in gene expression over the time course of infestation was expressed as the mean of all animals (n=6).

The final selection of potential BMs for evaluation based on the above criteria was on the availability of commercially produced antibodies for use in Western blotting due to the time constraints of the study.

2.2.3 Western Blotting

Western blotting was used as a method of determining if the available antibodies cross-reacted specifically with the relevant ovine BMs and to estimate if the selected BMs increased in relative concentration within sheep serum as the sheep scab infestation progressed. The detection of potential BM proteins identified by Western blotting was by chemiluminescence (GE Healthcare) and visualisation was on X-ray plates or using an ImageQuant LAS4000 camera (GE Healthcare, UK) available during the latter stages of the project. Blots visualised by ImageQuant are specified in the results.

2.2.3.1 Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed using a total sample volume of 10µl, after the sheep sera samples (diluted 1:10 in dH_2O) had been incubated with 4X NuPage LDS sample buffer (2.5µl) (Invitrogen) and NuPage sample reducing agent (1µl) (Invitrogen) at 70°C for 10 mins. Electrophoresis was performed using NuPage BisTris 4-12% Polyacrylamide gels (Invitrogen) using MES buffer described in A2.1.1 at 250V for 35 mins in a PowerEase 500 electrophoresis cell (Invitrogen). The molecular weight marker was 1X SeeBlue Prestained Standard (5µl) (Invitrogen) for X-ray development and MagicMark XP (5µl) (Invitrogen) for ImageQuant analysis.

2.2.3.2 Blotting

The Invitrogen iBlot Western Blotting System (programme P2) was used to transfer the separated proteins onto a nitrocellulose membrane (Invitrogen) which was then blocked with 3% gelatin (diluted using the wash buffer/antibody diluent described in A2.1.2.) from cold water fish skin (Sigma-Aldrich) for 30 minutes. Washing between all steps employed the wash buffer described in A2.1.2. Primary and secondary antibody incubations were performed for 1 hr at room temperature (RT).

2.2.3.3 Antibodies

Specific primary antibody information for each candidate protein is shown in Table 2.4. Each primary antibody was used at a dilution of 1:1000 as determined by titration blots, except the antibody for TNF which was used at a 1:500 dilution. Antibody diluent was as described in section A2.1.2. The secondary antibodies used with each blot are detailed in Table A2.1.

2.2.3.4 Controls

Negative controls (no primary antibody controls) were used with each Western blot to assess non-specific antigen interactions with the secondary antibodies. IgG controls using rabbit anti-sheep IgG HRP (Dako), at a dilution of 1:20000, were used instead of positive controls as there were no defined standards, i.e. commercially available recombinants, or sera available with known measurable amounts of the proteins being investigated. The IgG control was designed to recognise IgG in ovine sera thereby indicating that the Western blotting technique was working. The exception was C4BPB where a commercially available human recombinant protein was used as a positive control (H00000725-P01, Novus Biologicals).

2.2.3.5 ECL visualisation and image development

Western blots were visualised using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) following the manufacturer's instructions and the blot transferred to X-ray film with 30s exposure prior to development. Development and fixing of film was by automatic tank (Optimax 2010) and band densitometry was based on band volume as determined by Quantity One software (version 4.6.2, Bio-Rad). C4BPB visualisation was achieved using the ImageQuant system (LAS 4000, GE Healthcare) followed by band densitometry analysis by ImageQuant TL software (GE Healthcare).

2.2.3.6 Samples

The pooled sera samples separated by SDS-PAGE in the initial screening of the candidate proteins were obtained from three experimental *P. ovis* infestation trials comprised of the following:

1. A time course experimental study (as described in Burgess et al., 2012a) where weekly serum samples were taken from 6 sheep infested with *P. ovis* over a 6 week period and included pre-infestation samples (week 0). Lesion areas were recorded weekly when blood samples were taken. This study is referred to throughout as time course (TC)1.

2. A sheep scab experimental trial with 8 sheep blood sampled by venous extraction weekly (weeks 0-6). This study is referred to throughout as TC2.

3. A group of 6 mite donor sheep experimentally infested and blood sampled pre-infestation then weekly for 6 weeks. This study is referred to as TC3.

In addition, pooled hyper-immune (HI) sera (n = 2) was used from sheep 6 weeks post-infestation in a secondary infestation with *P. ovis* mites.

2.2.3.7 IgG depletion of sera

Initial blots (not shown) were difficult to interpret due to the presence of large bands of cross-reactivity at approximately 25 and 50 kDa. As this was likely to be IgG light and heavy chains, all sera used for blotting were IgG depleted using a NAb™ Protein G Spin Kit 0.2ml (Thermo Scientific) following the manufacturer's instructions. All serum used in SDS-PAGE was IgG depleted as this produced blots with reduced cross-reactivity which allowed clearer interpretation of results.

2.3 Results

2.3.1 Bioinformatic analysis

The original list of 621 differentially expressed genes was sorted in relation to time intervals (week post-infestation). The numbers of genes within each interval of the time groups were as follows: 0 *vs.* 1 = 36; 1 *vs.* 3 = 66; 0 *vs.* 3 = 51; 3 *vs.* 6 = 403; 0 *vs.* 6 = 606; and 1 *vs.* 6 = 465. Many of the genes were present in more than one list hence the cumulative numbers exceeding the 621 original genes.

The number of genes in each interval list at each stage of the filtering process (as described in section 2.2.2) are depicted in a flow diagram (Figure 2.2) to demonstrate how the filtering process progressed from 621 genes in the original list to 178 filtered genes in the final list.

The Genespring "entity inspector" function re-assessment, as described in section 2.2.2, was not included in Figure 2.2 as no further genes were excluded during this step of the analysis. This function considered the strength of the relationship between the time course of infestation and individual gene expression; lesion size changes and changes in gene expression, and the magnitude of the gene expression changes over the total time of infestation. An example of the "entity inspector" function analysis is shown in Figure 2.3.

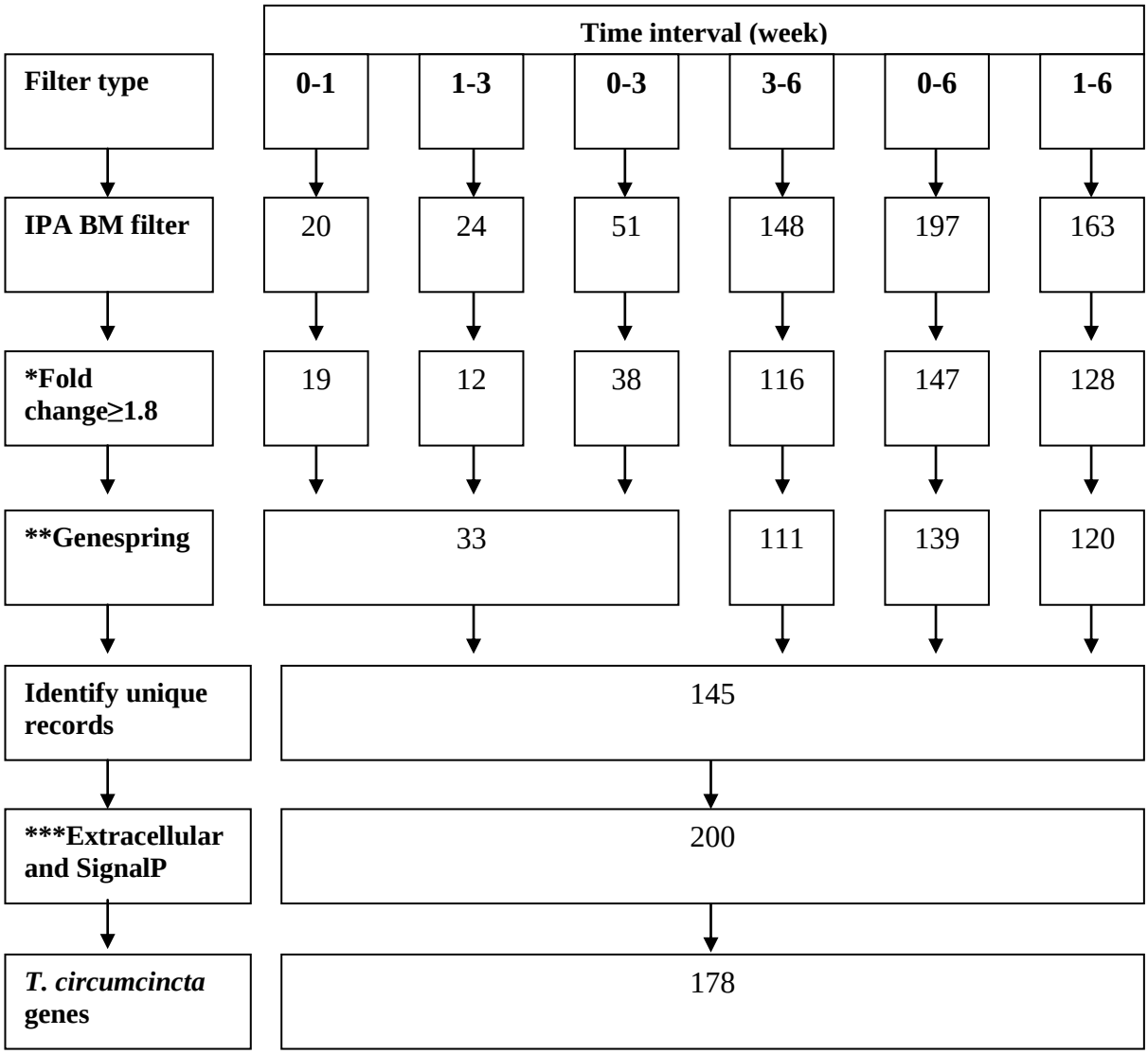

Figure 2.2: Flow diagram demonstrating gene numbers after each filtering process as described in section 2.2.2 and outlined in Figure 2.1. *Only up-regulated genes were used in the final analysis. **"Find similar entities" function (Genespring GX). ***Extra genes added in from those encoding proteins with extracellular locations and SignalP peptides.

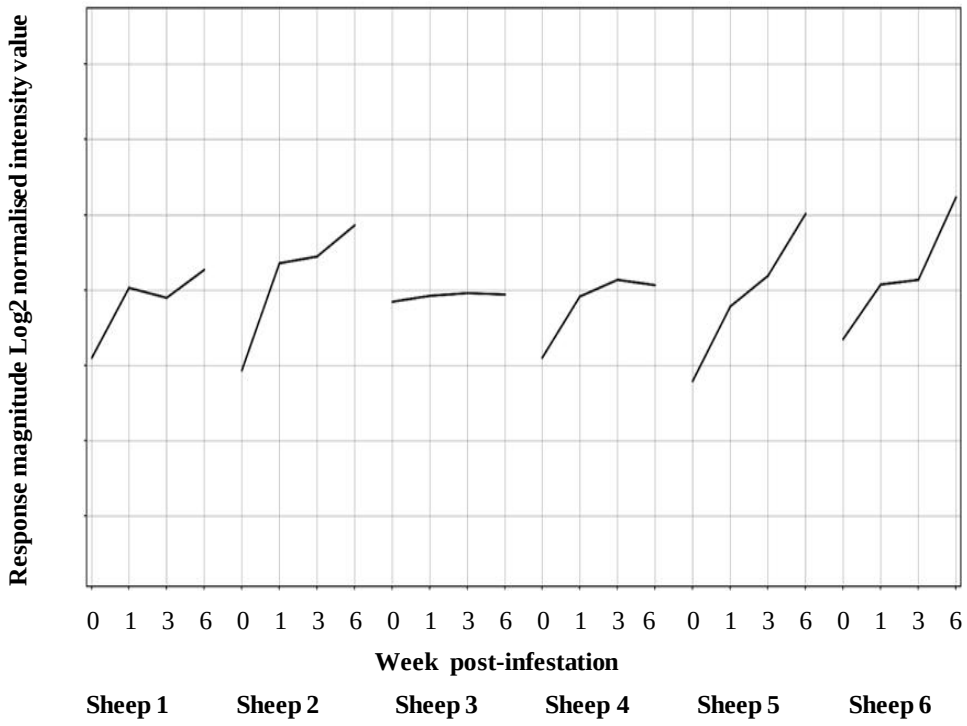

Figure 2.3: An example of an entity inspector analysis of a high ranking gene response showing gene expression magnitude responses across all animals and for all time points for collectin11 (*COLEC11)*. Response magnitude measured as log2 normalised intensity values. Week p.i. = week post-infestation with *P. ovis.* Diagram adapted from Genespring GX.

The protein encoded by *COLEC11* has functions within the immune system and was considered to be a highly ranked potential BM as the gene expression responses increased for all time points in all animals with expanding lesions over time (see Table 2.1 for measured lesion sizes for individual sheep across the time course of infestation) and the animals with the largest lesions, (sheep 2, 5 and 6), showed the greatest response magnitudes.

Table 2.1: Lesion sizes (cm^2) across the time course of sheep scab infestation for all sheep (n=6) from the trial which generated the data for the transcriptomic analysis of circulating leukocytes (Burgess et al., 2012a). R= naturally resolving lesion.

Sheep no	**Week post – infestation**							
	0	**1**	**2**	**3**	**4**	**5**	**6**	**R**
1	0	25	50	100	200	100	50	Yes
2	0	6	100	150	250	300	600	
3	0	16	6	6	4	0	0	Yes
4	0	4	16	100	25	32	0	Yes
5	0	12	150	400	800	1800	3600	
6	0	10	150	300	800	2400	2500	

The function of the final assessment using the "entity inspector" (Genespring GX), was to allow annotation of the BM and data quality filtered genes according to the three scores described as "time tracking score", "response magnitude (Log2)" and "lesion tracking score" in Table 2.2. In this table, potential BMs, from the highest ranking (i.e. best "time tracking" score which represents the effectiveness of each gene to reflect *P. ovis* infestation over time) to the lowest ranking are presented. The "time tracking score", as opposed to the "response magnitude" or "lesion tracking score", was used for ranking purposes (Table 2.2) as this factor was considered as having potentially wider application in the field i.e. all sheep scab cases develop over a certain time period, but the extent of the disease and lesion size shows more individual variation.

HUGO Gene Symbol[A]	Blood[B]	Plasma/Serum[B]	Saliva[B]	Sputum[B]	Data Quality Verified[C]	Location[D]	Time Tracking Score[E]	Response Magnitude (Log2) [F]	Lesion Tracking Score[E]
TRPC6	x	x			Yes		1	2.9	2
RNASE6					Yes	Signal Peptide	1	2.6	3
KCNQ1	x				Yes	Signal Peptide	2	1.2	2
ADORA3	x				Yes		2	1.2	2
COLEC11	x	x			Yes		2	2.5	2
HK3	x	x			Yes		2	0.9	2
MPDZ	x	x			Yes		2	2.2	2
OXT	x	x			Yes		2	1.5	2
PDLIM1	x				Yes		2	3.5	2
RNASE4				x	Yes		2	1.1	2
SLC7A11	x	x			Yes		2	1.7	2
TKT	x	x			Yes		2	0.7	2
ICAM3	x	x			Yes		2	2.0	3
CCDC85A					Yes	Signal Peptide	2	1.4	3
FFAR2					Yes	Signal Peptide	2	2.6	3
METRNL					Yes	Signal Peptide	2	0.9	3
RAMP2					Yes	Signal Peptide	2	1.2	3
VSTM1					Yes	Signal Peptide	2	1.1	3
ADRB2	x				Yes		2	0.9	3
C4BPB	x	x			Yes		2	2.8	3
CAMK1	x	x			Yes		2	2.1	3
CSF2RB	x				Yes		2	0.9	3
FAM107A	x	x			Yes		2	1.6	3
KIAA0649	x	x			Yes		2	1.2	3
MYB	x				Yes		2	1.8	3
PLA2G7	x				Yes		2	1	3
SUCNR1	x				Yes		2	3	3
TBC1D16	x	x			Yes		2	1.3	3
C11ORF24					Yes	Extracellular	2	0.6	4

HUGO Gene Symbol[A]	Blood[B]	Plasma/Serum[B]	Saliva[B]	Sputum[B]	Data Quality Verified[C]	Location[D]	Time Tracking Score[E]	Response Magnitude (Log2) [F]	Lesion Tracking Score[E]
EMB					Yes	Signal Peptide	2	0.5	4
HEMK1					Yes	Signal Peptide	2	0.7	4
MYADM					Yes	Signal Peptide	2	0.7	4
CKB	x	x			Yes		2	0.7	4
CSNK1E	x				Yes		2	0.8	4
GAPDH	x	x		x	Yes		2	0.6	4
GGA2	x	x			Yes		2	0.8	4
HNRNPL	x	x			Yes		2	1	4
MYH4	x				Yes		2	1.7	4
NFATC3	x	x			Yes		2	0.8	4
TOR2A	x	x			Yes		2	0.8	4
SCAP					No	Signal Peptide	2	0.7	5
MAPK7	x	x			Yes		2	1.6	5
ABCD1					Yes	Signal Peptide	3	0.8	2
NLRP7	x	x			Yes		3	1.7	2
S100A11	x			x	Yes		3	0.9	2
CCDC19					Yes	Signal Peptide	3	1.4	3
EMR3					Yes	Signal Peptide	3	0.2	3
GRIN2D					Yes	Signal Peptide	3	0.9	3
HIPK2					Yes	Signal Peptide	3	1.2	3
IGSF9					Yes	Signal Peptide	3	1.7	3
NCLN					Yes	Signal Peptide	3	0.9	3
NT5M					Yes	Signal Peptide	3	1.2	3
PERP					Yes	Signal Peptide	3	1	3
RAB3GAP2					Yes	Signal Peptide	3	0.9	3
SFXN5					Yes	Signal Peptide	3	0.9	3
SEPT5	x				Yes		3	1.9	3
C3AR1	x				Yes		3	1.5	3
CASP3	x				Yes		3	1	3

HUGO Gene Symbol[A]	Blood[B]	Plasma/Serum[B]	Saliva[B]	Sputum[B]	Data Quality Verified[C]	Location[D]	Time Tracking Score[E]	Response Magnitude (Log2) [F]	Lesion Tracking Score[E]
CD63	x				Yes		3	1.2	3
DOK2	x				Yes		3	0.8	3
GLIPR2	x				Yes		3	0.5	3
IL18RAP	x				Yes		3	1.5	3
MRC2	x	x			Yes		3	1.3	3
MSRA	x				Yes		3	0.8	3
PRAM1	x				Yes		3	1.5	3
PTPN1	x				Yes		3	0.9	3
RASGRP4	x				Yes		3	0.9	3
SIRPA	x				Yes		3	0.6	3
STXBP2	x				Yes		3	0.6	3
TNFSF14	x				Yes		3	0.7	3
AIG1					Yes	Signal Peptide	3	0.7	4
C11orf24					Yes	Signal Peptide	3	0.6	4
C22orf25					Yes	Signal Peptide	3	0.7	4
ESRRA					Yes	Signal Peptide	3	0.6	4
HEATR7A					Yes	Signal Peptide	3	0.8	4
KCNK7					Yes	Signal Peptide	3	0.8	4
NDUFB11					Yes	Signal Peptide	3	0.8	4
TMED3					Yes	Signal Peptide	3	0.6	4
ACAA1	x				Yes		3	0.8	4
ALDH2	x	x			Yes		3	0.7	4
CAMK2G	x	x			Yes		3	0.9	4
CD244	x				Yes		3	1.2	4
CDKL5	x	x			Yes		3	1.5	4
CNR2	x				Yes		3	0.6	4
CTSF	x				Yes		3	0.7	4
DGKZ	x	x			Yes		3	0.7	4
FBXW5	x	x			Yes		3	0.7	4

HUGO Gene Symbol[A]	Blood[B]	Plasma/Serum[B]	Saliva[B]	Sputum[B]	Data Quality Verified[C]	Location[D]	Time Tracking Score[E]	Response Magnitude (Log2) [F]	Lesion Tracking Score[E]
GCSH	x	x			Yes		3	0.7	4
GPX4	x	x			Yes		3	0.7	4
LSP1	x				Yes		3	0.6	4
LTA4H	x				Yes		3	0.8	4
PLEKHM2	x	x			Yes		3	0.7	4
POLD3	x	x			Yes		3	0.6	4
PPEF2	x	x			Yes		3	1.5	4
RRP12	x	x			Yes		3	0.5	4
SF3B1	x	x			Yes		3	1.2	4
SLA	x				Yes		3	0.9	4
VASP	x				Yes		3	0.4	4
ADAMTS13					Yes	Signal Peptide	3	1.1	5
RAB26					Yes	Signal Peptide	3	1.3	5
COL4A3BP	x				Yes		3	0.9	5
ITGA2B	x	x			Yes		3	0.7	5
ITGAE	x	x			Yes		3	1	5
MDM2	x				Yes		3	0.8	5
SPP1	x	x			Yes		4	3	2
MBOAT7					Yes	Signal Peptide	4	0.9	3
PAK1	x				Yes		4	0.5	3
PTAFR	x				Yes		4	0.7	3
TALDO1				x	Yes		4	0.7	3
ART5					Yes	Signal Peptide	4	1.2	4
CLCN7					Yes	Signal Peptide	4	0.7	4
ITGA2B					Yes	Signal Peptide	4	0.8	4
SLC13A3					Yes	Signal Peptide	4	0.6	4
TMEM185A					Yes	Signal Peptide	4	0.7	4
TSPO					Yes	Signal Peptide	4	0.8	4
ARID3A	x	x			Yes		4	0.6	4

HUGO Gene Symbol[A]	Blood[B]	Plasma/Serum[B]	Saliva[B]	Sputum[B]	Data Quality Verified[C]	Location[D]	Time Tracking Score[E]	Response Magnitude (Log2) [F]	Lesion Tracking Score[E]
ARRB1	x				Yes		4	0.7	4
CD34	x				Yes		4	1.5	4
CDK5R1	x				Yes		4	1.5	4
HMOX2	x	x			Yes		4	0.6	4
NCR3	x				Yes		4	0.7	4
OSBPL1A	x				Yes		4	0.5	4
PRKCA	x				Yes		4	1	4
SOS2	x	x			Yes		4	0.8	4
TIMP2	x	x			Yes		4	1	4
PSMA7	x	x			Yes		4	1.8	5
CXADR					Yes	Signal Peptide	5	0.8	3
ECE1	x	x			Yes		5	0.6	3
HSH2D	x				Yes		5	0.6	3
MYO5B	x	x			Yes		5	1.2	3
PIGR	x	x		x	Yes		5	1.8	3
SIVA1	x				Yes		5	0.6	3
TGFA	x		x		Yes		5	0.8	3
TPM1	x				Yes		5	0.1	3
CHMP1A					Yes	Extracellular	5	0.7	4
CDH4					Yes	Signal Peptide	5	0.8	4
FAM174A					Yes	Signal Peptide	5	0.9	4
FBXW7					Yes	Signal Peptide	5	0.7	4
RELT					Yes	Signal Peptide	5	0.6	4
RNF144B					Yes	Signal Peptide	5	0.5	4
TP53I11					Yes	Signal Peptide	5	0.8	4
ALPL	x	x			Yes		5	0.6	4
BASP1	x	x			Yes		5	0.6	4
CD97	x				Yes		5	0.6	4
CYBA	x				Yes		5	1	4

HUGO Gene Symbol[A]	Blood[B]	Plasma/Serum[B]	Saliva[B]	Sputum[B]	Data Quality Verified[C]	Location[D]	Time Tracking Score[E]	Response Magnitude (Log2) [F]	Lesion Tracking Score[E]
ECM1	x	x			Yes		5	1.6	4
FAM129A	x	x			Yes		5	0.7	4
FGR	x				Yes		5	0.4	4
IGF1R	x				Yes		5	0.6	4
LRG1	x	x			Yes		5	0.1	4
NRG1	x				Yes		5	0.2	4
RPN1	x	x			Yes		5	0.7	4
S100A8	x	x	x	x	Yes		5	1	4
S100A9	x	x		x	Yes		5	0.9	4
SELPLG	x	x			Yes		5	0.6	4
SOCS1	x				Yes		5	0.7	4
TCN1	x	x	x		Yes		5	0.5	4
CA4					Yes	Signal Peptide	5	0.2	5
CCNJL					Yes	Signal Peptide	5	0.3	5
EXOC8	x	x			Yes		5	1	5
MAN1A2	x	x			Yes		5	0.9	5
MPO	x	x		x	Yes		5	0.9	5

Table 2.2: Final list of 178 filtered and selected BMs. All genes were considered to be significantly differentially expressed with a p-value of ≤0.05 following Benjamini & Hochberg FDR correction for multiple testing. HUGO gene symbols obtained from HUGO gene nomenclature committee (HGNC). The gene symbols used were ovine where available and otherwise bovine, or human if the bovine symbol was not available. [B]Protein that HUGO gene encodes for was identified from the Ingenuity Pathway Analysis Knowledge Database

as being present in the selected biofluid. [x]Represents protein is present in biofluid. [C]Raw data and quality flags checked for each gene as described in section 2.2.2. [D]Location column denotes either the normal location of the protein encoded by the represented gene where not previously identified in a particular biofluid, or the presence of a predicted signal peptide sequence (identified from SignalP 3.0 package). [E]Score assigned for how closely the mean gene expression profile tracked the course of infestation over time or how the individual gene expression profile tracked the lesion size over time (1 = excellent; 2 = very good; 3 = good; 4 = average; 5 = poor). [F]Range of log normalised intensity value (Log2) for individual genes based on mean expression profile over all animals over the time course of infestation. Blue highlighted genes = encoding proteins for which commercially-available antibodies could be obtained (Table 2.3).

The final selection of potential BMs for downstream analysis was based on the availability of commercially produced antibodies for the detection of orthologues of the highest ranking proteins and included 8 proteins which are listed in Table 2.3 with antibody source details and highlighted in blue in Table 2.2.

Table 2.3: The final list of potential protein BMs for downstream analysis for which commercially available antibodies could be sourced at the time of study. *HUGO gene symbols as approved by the HGNC.

Candidate protein	Gene symbol*	Antibody	Source	Catalogue No
Osteopontin	*SSP*	Rabbit α-human	Abcam	Ab8448
Calgranulin-A	*S100A8*	Mouse α-human	Novus Biological	A01
Calgranulin-B	*S100A9*	Rabbit α-human	Abcam	Ab75478
Complement 4 binding protein	*C4BPB*	Mouse α-human (monoclonal)	Abnova	H00000725-M01
	C4BPB	Mouse α-human (polyclonal)	Abnova	H00000725-A01
Oxytocin	*OXT*	Guinea-pig α-human	Abcam	Ab6908-1
Potassium voltage gated channel	*KCNQ1*	Rabbit α-human	Abcam	Ab77701
Solute carrier family 7	*SLC7A11*	Rabbit α-human HRP	Abcam	Ab81948
Collectin 11	*COLEC11*	Rabbit α-human	Abcam	Ab91483

In the sheep scab host gene expression study (Burgess et al., 2012a) which provided the data used in the BM search described in this chapter, the microarray gene expression data were verified using qPCR. It was necessary to perform qPCR validation as a routine quality assurance step but also because the microarray probes were derived from bovine gene sequence data, as ovine specific arrays were not available at the time of study.

Table 2.4: Correlations between the microarray analysis and qPCR results for 10 genes included in the bioinformatic analysis. Comparisons shown are between the baseline samples (time = 0) and the individual time point samples (WPI = week post-infestation). *Correlation between qPCR and microarray data for selected validation candidates denoted by the Pearson r value. Table adapted from Burgess et al (2012a).

Gene Symbol	**Microarray fold change**			**qPCR fold change**			**Pearson r***
	1 WPI	3 WPI	6 WPI	1 WPI	3 WPI	6 WPI	
SPP1	2.18	3.27	53.33	5.98	4.48	12.42	0.98
ALAS2	-1.45	-15.48	-59.17	-1.57	-44.07	-256.53	0.99
ALOX15	3.1	5.76	17.47	3.43	6.19	7.93	0.89
IGFBP4	2.23	4.26	12.62	1.45	2.87	2.13	0.57
MPO	1.13	-2.54	1.05	1.58	-3.40	-3.12	0.56
C4BPB	2.37	3.56	11.92	2.89	2.25	3.03	0.55
CTTN	2.23	3.18	3.11	1.53	2.05	1.56	0.66
PDLIM	4.11	7.21	52.5	6.16	7.65	16.63	0.99
PLAU	1.59	2.26	6.34	1.08	1.56	2.43	0.97
ANG	1.76	2.91	10.48	-1.46	-1.15	1.95	0.99
Mean correlation							**0.82**

Ten genes were selected from the microarray study (Table 2.4) and the mean Pearson correlation value across all genes between the microarray and qPCR data was shown to be 0.82. This demonstrated that the two methods of assessing differential gene expression showed close agreement, giving increased confidence in the microarray results and as such the array data was considered to be validated (Burgess et al., 2012a).

As there were no ovine specific antibodies available for the assessment of potential BMs, ovine or if not available, bovine amino acid sequences for each of the proteins to be evaluated were aligned with the corresponding human amino acid sequence to indicate identities between the sequences and therefore if cross-reactivity between them was likely. The results are shown in Figure A2.1 and a summary (sequence % identities) included in Table 2.5. An additional two proteins, tumour necrosis factor alpha (TNF) and tenascin C (TNC) were added to the list of potential BMs (Table 2.6) due to their up-regulated gene response to *P. ovis* infestation in ovine skin (Burgess et al., 2010).

Table 2.5: Amino acid sequence alignment identities of potential BM proteins with the species the antibody to be used was raised against. The full sequence alignments are included in Figure A2.1. If the ovine sequence was not available the bovine sequence was used. *No full ovine or bovine sequence available so the sequenced fragment was aligned with the corresponding fragment of the human protein.

Protein to be evaluated	Ovine amino acid % identity against human	Bovine amino acid % identity against human	Accession number (UniProt, EMBL-EBI)
Osteopontin (SPP)	58		Human P10451 Ovine Q9XSY9
Calgranulin A (S100A8)		69	Human P05109 Bovine P28782
Calgranulin B (S100A9)		51	Human P06702 Bovine P28783
Complement 4 Binding Protein (C4BPB)		49	Human P20851 Bovine Q28066
Tumour Necrosis Factor-α (TNF)		93 (bovine) 93 (ovine)	Bovine Q06599 Ovine P23383
Oxytocin (OXT)	88		Human P01178 Ovine P13389
Potassium voltage gated channel (KCNQ1)		88 *	Human P51787 Bovine F1N4R0
Solute carrier family 7 (SLC7A11)		98 *	Human Q9UPY5 Bovine B6D7N9
Collectin 11 (COLEC11)		84	Human Q9BWP8 Bovine Q17QH6
Tenascin C (TNC)		68	Human P24821 Bovine A0JN60

Table 2.6: Additional potential BMs for downstream analysis for which commercially available antibodies could be sourced at the time of study. *Human genome organisation (HUGO) gene symbols as approved by the HUGO gene nomenclature committee (HGNC). *antibody raised against recombinant bovine TNF. **antibody raised against a fusion protein, corresponding to amino acids 18-175 of human TNC.

Candidate protein	HUGO gene symbol*	Primary Antibody	Source	Catalogue No
Tumour necrosis factor alpha	*TNF*	Mouse α-bovine*	ABD Serotec	MCA23358
Tenascin C	*TNC*	Chicken α-human**	Abcam	Ab16290

2.3.2 Western Blots of selected proteins

2.3.2.1 Osteopontin (SPP)

The primary antibody used (Table 2.3) had been shown to react with human, rat, dog and pig SPP according to the manufacturer's data sheet (Abcam), but had not been previously tested with ovine SPP. Bands at 35.4 kDa on the Western blot of the sheep sera were not detected at any point pre or post infestation on repeated blots and it is therefore likely that either the human specific SPP antibody used did not bind ovine SPP, or SPP was present in very low abundance in sheep sera.

2.3.2.2 Calgranulin (S100A8 and S100A9)

There were no bands present at 16kDa, the estimated MW for S100A9. The antibody used to detect ovine S100A9 had been indicated in the manufacturer's data sheet

(Abcam) to cross-react with human, mouse and rat S100A9, but had not been previously tested with ovine S100A9. No specific band for S100A8 was detected at the expected MW of 11 kDa (Figure 2.4). This antibody had only been previously tested against human S100A8 as indicated by the manufacturer's data sheet (Novus Biologicals). Bands in the negative control (lane 11) indicated non-specific binding between the sheep sera and the secondary antibody. As with SPP, it is likely that either the antibodies used in the detection of S100A9 and S100A8 did not bind with the ovine proteins, or the proteins were present in low concentrations in sheep sera.

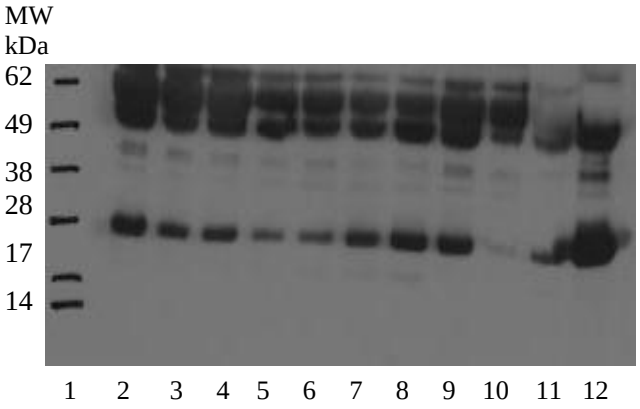

Figure 2.4: X-ray image of a Western blot using pooled sera (n = 6) from sheep infested with *P. ovis* over a 6 week time course (TC1) run on a SDS-PAGE gel. Electrophoresis was performed at 250V for 35 mins. Western blot was probed with a S100A8 antibody raised in mice against human rS100A8. Conjugate = rabbit anti-mouse immunoglobulins HRP. Lane 1 = SeeBlue MW Marker (Invitrogen); 2 = pre-bleed sera; 3 = week 1 pi; 4 = week 2 pi; 5 = week 3 pi; 6 = week 4 pi; 7 = week 5 pi; 8 = week 6 pi; 9 = hyper-immune (HI) sera; 10 = blank lane; 11 = negative (-ve) (no primary antibody) control; 12 = positive control. Positive control (+ve) used pooled 6 week pi ovine sera (as in lane 8) probed with rabbit anti-ovine IgG HRP (Dako).

2.3.2.3 Complement 4 binding protein beta chain (C4BPB)

C4BPB was initially detected using a polyclonal antibody raised in mice against human rC4BPB, but this yielded indistinct bands at 28 kDa, the estimated MW for human C4BPB. The signal was improved when a monoclonal anti-C4BPB antibody raised in mice against human rC4BPB was used (Table 2.3). The results indicated that a protein of the expected MW (28 kDa) showed increasing relative concentration (as measured by band density) in weeks 5 and 6 post-infestation (Figure 2.5). The band obtained pre-infestation was of a higher band density than weeks 1-4 post-infestation, which may be explained as the pooled sera used in this blot (TC1) came from sheep which had been skin biopsied at the same time the pre-bleed was taken (Burgess et al., 2010), thereby potentially increasing serum inflammatory proteins. The negative control (lane 9) was blank indicating that there was no non-specific interactions between the antigen and the secondary antibody and the positive control (human rC4BPB) gave an intense band at the expected MW of 28 kDa, suggesting that the bands obtained at 28 kDa were likely to be ovine C4BPB.

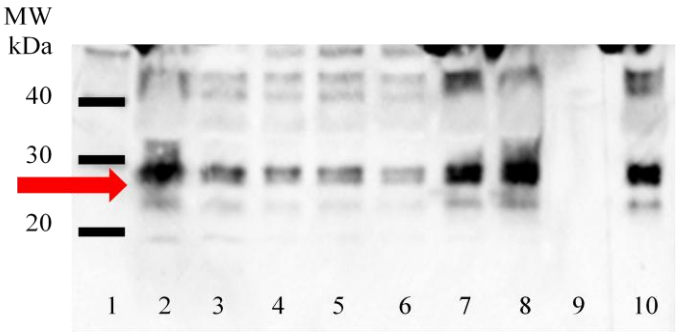

Figure 2.5: Image of a Western blot from a SDS-PAGE gel using sera (pooled n = 6) from sheep infested with *P. ovis* over a 6 week time course (TC1) probed with a monoclonal anti-C4BPB (mC4BPB) antibody raised in mice against human rC4BPB. The band of interest at 28kDa is indicated by a red arrow. Lane 1 = MW marker MagicMark XP (Invitrogen); 2 = pre-bleed sera; 3 = week 1 pi; 4 = week 2 pi; 5 = week 3 pi; 6 = week 4 pi; 7 = week 5 pi; 8 = week 6 pi; 9 = -ve (no primary antibody) control; 10 = +ve control using human rC4BPB (Abnova). Conjugate used was rabbit anti-mouse Ig HRP (Dako). Visualisation was by ECL Plus (GE Healthcare) and ImageQuant camera LAS4000 (GE Healthcare).

As lesion size data was available for the TC1 infestation study, separate Western blots were performed using individual sera from two sheep from this trial. One of these sheep had a naturally resolving lesion throughout the 6 week infestation trial (Sheep 1 in Table 2.1 and labelled Sheep 1 in Figure 2.6 below) whilst the other animal had an expanding lesion (Sheep 5 in Table 2.1 and labelled Sheep 5 in Figure 2.6 below). The results of the densitometry analyses of these blots (Figure 2.6) indicated that as the lesion size increased, the 28 kDa band increased in intensity and as the lesion resolved, the intensity of the band decreased.

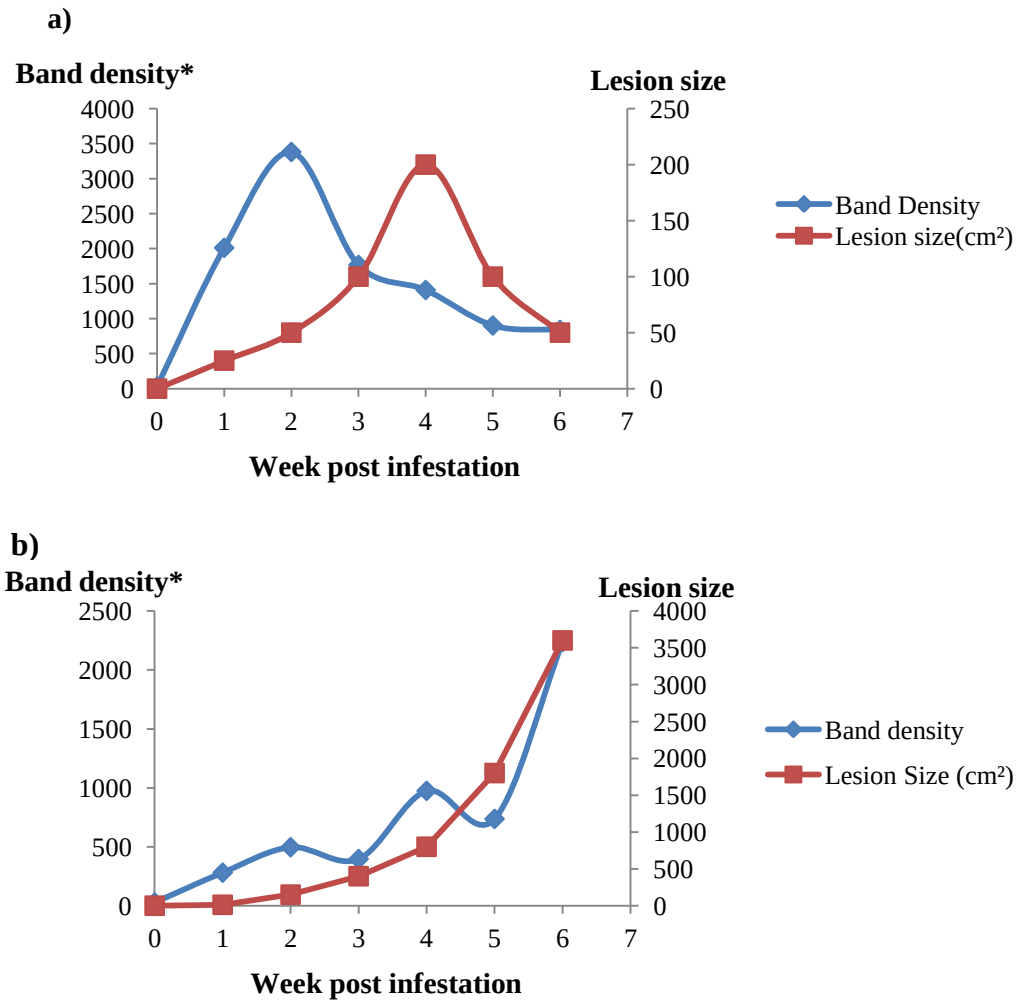

Figure 2.6: Densitometry analysis (ImageQuant) from Western blot images using: **a)** serum (TC1) from a sheep infested with *P. ovis* where the lesion resolved naturally (Sheep 1). **b)** serum from an infested sheep (TC1) where the lesion had expanded over the 6 week time course (Sheep 5). The immunoblots were probed with a monoclonal antibody raised in mice against human rC4BPB (Abnova) and the conjugate used was rabbit anti-mouse immunoglobulin HRP (Dako). The image was visualised by ECL Plus and ImageQuant and band densities (at 28 kDa) were quantified by ImageQuant analysis.

2.3.2.4 Tumour necrosis factor alpha (TNF)

A problem commonly found in Western blot analysis is cross-reactivity between samples and secondary antibodies (Signore and Reeder, 2012) indicated by bands in the negative control as seen in lane 11, Figure 2.4 (S100A8). A biotinylated primary antibody and a streptavidin enzyme conjugate were available for the detection of TNF, therefore negating the need for a secondary antibody and eliminating the possibility of secondary antibody cross-reactivity. The biotinylated anti-TNF antibody used was raised in mice against bovine TNF and was predicted according to the manufacturer (ABD Serotec) to react with ovine TNF. Bands were obtained at the expected molecular weight of 25.6 kDa, marked with a red arrow as shown in Figure 2.7, but as there was no positive control available to confirm the identity of the bands, and it is unlikely that the bands would remain at a similar high density throughout the 6 weeks of infestation, it is possible that the antibody may have cross-reacted with ovine IgG in the sera sample.

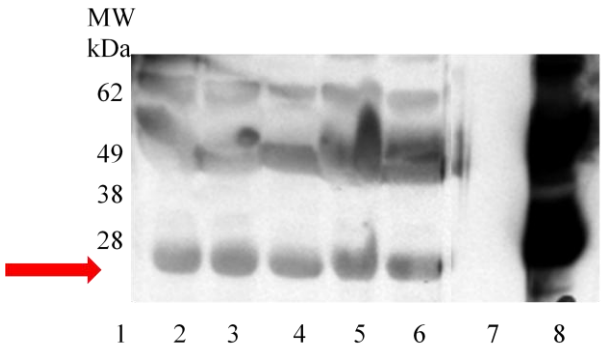

Figure 2.7: X-ray image of a Western blot using pooled sera (n = 6) from sheep infested with *P. ovis* over a 6 week time course (TC1) probed with a biotinylated antibody raised in mice against bovine TNF. Lane 1= SeeBlue MW Marker (Invitrogen); 2 = pre-bleed sera; 3 = week 2 pi; 4 = week 3 pi; 5 = week 5 pi; 6 = week 6 pi; 7 = –ve control (no primary antibody); 8 = +ve (sheep IgG) control.

2.3.2.5 Oxytocin (OXT)

The results from the Western blot analysis, using a polyclonal anti-OXT antibody raised in guinea-pigs against human OXT, indicated no 12.7kDa band in pre-bleed sera but faint bands were present at weeks 1, 3 and 6 p.i., although these bands did not appear to increase in intensity over time (Figure 2.8). The multiple bands obtained in all lanes apart from the negative control suggested that the antibody was binding to multiple proteins in the sheep sera.

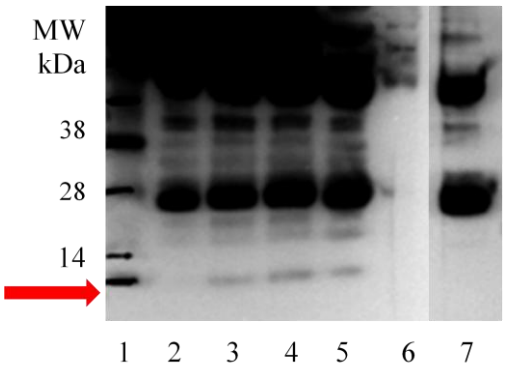

Figure 2.8: X-ray image of Western blot using pooled sera (n = 6) from sheep infested with *P. ovis* over a 6 week time course (TC1) probed with a primary antibody raised in guinea-pig against human OXT. Lane 1= SeeBlue MW Marker (Invitrogen); 2 = pre-bleed sera; 3 = week 1 pi; 4 = week 3 pi; 5 = week 6 pi; 6 = -ve control; 7 = +ve (sheep IgG) control

2.3.2.6 Potassium voltage gated channel (KCNQ1)

No bands at the predicted MW of 74.7 kDa were obtained for KCNQ1 following Western blot analysis. This antibody was predicted to react with bovine KCNQ1 according to the manufacturer (Abcam), suggesting it may be suitable for use with ovine sera, but either it did not react, or KCNQ1 was not present in sufficient quantities in the sheep sera to be detected.

2.3.2.7 Solute carrier family 7 (SLC7A11)

This protein was highly ranked following the filtering process and an HRP linked primary antibody was available which negated the use of a secondary antibody. The Western blot showed a band present at 55.4 kDa, the predicted MW of SLC7A11, but the band intensities appeared very similar pre-infestation and throughout the time

course of infestation, with a slight increase by week 6 post-infestation and also with hyper-immune sera as shown in Figure 2.9.

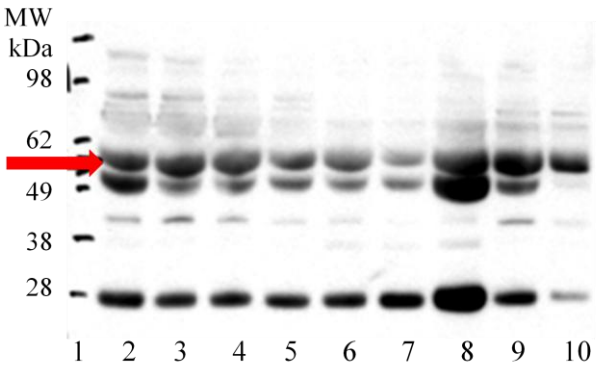

Figure 2.9: X-ray image of a Western blot using pooled sera (n = 6) from sheep infested with *P. ovis* over a 6 week time course (TC1) probed with a HRP labelled primary antibody raised in rabbits specific to human SLC7A11. Lane 1= SeeBlue MW Marker (Invitrogen); 2 = pre-bleed sera; 3 = week 1 pi; 4 = week 2 pi; 5 = week 3 pi; 6 = week 4 pi; 7 = week 5 pi; 8 = week 6 pi; 9 = HI sera; 10 = +ve (sheep IgG) control.

2.3.2.8 Tenascin C (TNC)

Tenascin C was not detected at the predicted MW of 241 kDa. The antibody was predicted to react with human, mouse and rat Tenascin C protein according to the manufacturer (Abcam), but had not been tested in other species including sheep. It is likely, in the absence of bands at the estimated MW of TNC that the antibody used, which was raised in chickens against human TNC, either did not bind the ovine protein or TNC was not present in sufficient quantities in sheep sera to be detectable.

2.3.2.9 Collectin 11 (COLEC11)

A faint band was evident at the predicted MW of COLEC11, 28.6 kDa, which did not appear to increase in intensity until weeks 5 and 6 post-infestation when a marginal increase was observed (Figure 2.10). The banding obtained in the negative control was less intense than appeared in any of the sample lanes which may indicate weak recognition, therefore COLEC11 should be re-tested when a bovine or ovine specific antibody becomes available.

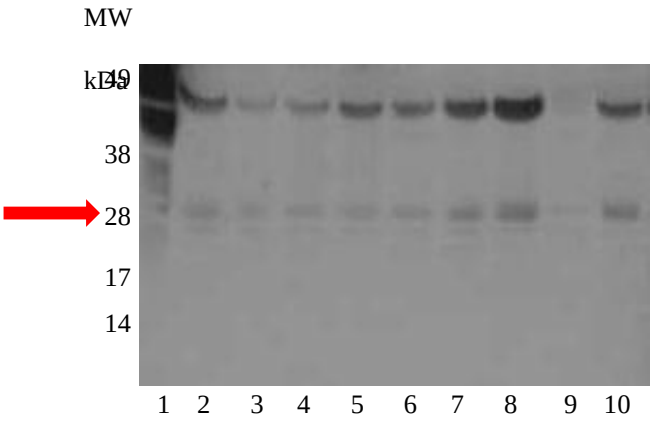

Figure 2.10: X-ray image of a Western blot using pooled sera (n = 6) from sheep infested with *P. ovis* (TC1) probed with an antibody raised in rabbits against human rCOLEC11. Conjugate = pig anti-rabbit HRP (Dako). Lane 1 = SeeBlue MW Marker (Invitrogen); 2 = pre-bleed sera; 3 = week 1 pi; 4 = week 2 pi; 5 = week 3 pi; 6 = week 4 pi; 7 = week 5 pi; 8 = week 6 pi; 9 = -ve (no primary antibody) control; 10 = +ve (sheep IgG) control.

2.4 Discussion

This chapter describes the identification of the BM potential of 178 genes from a group of 621 genes previously shown to be differentially expressed in ovine circulating leukocytes across a time course of sheep scab infestation (Burgess et al., 2012a), followed by the preliminary evaluation of the selected proteins, for which commercially available antibodies were available at the time of study, by Western blotting.

A potential issue with the use of gene expression analysis in the identification of diagnostic protein BMs, is that gene expression levels do not necessarily predict protein levels accurately (Verma et al., 2006). However, in biological systems, energy is generally conserved and it is therefore unlikely that an up-regulation in gene expression will not result in an up-regulation of transcribed protein, although there may be potentially a time delay between the two events. Protein assays are the method of choice for clinical diagnostic tests based on BMs but as proteomic technologies, such as mass spectrometry, are currently limited in terms of their protein separation capability (Kulasingam et al., 2010) genomic approaches are commonly adopted for the identification of novel BMs in clinical research. This is in part due to proteomics being at an earlier stage of development compared with genomic technologies but they are increasingly being used as complementary to a genomic approach and are useful as protein levels give a clearer indication of functional differences in cells and organs in health and disease (Verma et al., 2006). As such, a similar approach was adopted here in the search for BMs, i.e. potential BMs were identified at gene level and finally validated at protein level, to indicate

current disease status in sheep infested with *P. ovis*. Protein assays are also useful as they are commonly developed using easily accessible biofluids, such as blood, which makes sampling minimally invasive (Kulasingam et al., 2010). Many of the proteins transcribed from the highly ranked genes in this study (Table 2.2) are inflammatory plasma proteins, which originate from the liver, e.g. C4BPB is synthesised in hepatocyte cells. However, it is not practical to sample from tissue as part of a routine diagnostic test and as the blood circulation is the transport system of the mammalian body, it was anticipated that differences in inflammatory protein levels between healthy and diseased animals would be detected in blood.

The gene expression microarray analysis (Burgess et al., 2012a) on which the selection of potential BMs was formed, examined the changes in gene expression in circulating leukocytes across a time course of infestation with *P. ovis.* In sheep, blood leukocytes comprise neutrophils (40-60%), eosinophils (1-4%), basophils (0.4-1%), lymphocytes (25-35%) and monocytes (4-6%) (Alberts et al., 2007), the levels of which affects the levels of pro-inflammatory markers at sites of local inflammation as well as systemically. Leukocytes may reflect systemic and local events during an inflammatory response and can also influence the course of the inflammatory response at local tissue sites (Luster, 1998). The host response to infestation with *P. ovis* can be characterised as an initial immediate hypersensitivity-type response followed by a later systemic inflammatory response (Van den Broek and Huntley, 2003a). Increased leukocyte activation and extravasation occurs in response to up-regulation of the mediator selectin P ligand (*SELPLG)* (Burgess et al., 2012a) and recently it has been illustrated that as leukocytes interact and

communicate with every tissue in the body, they can be used as sentinels reflecting disease progression at a local level (Burgess et al., 2012a, Liew et al., 2006). However, it is not known if gene expression differences in leukocytes correlate to protein levels in serum, particularly as the main origin of inflammatory plasma proteins is in the liver not within circulating leukocytes. To minimise the effect of this, only proteins which were known to be secreted or had an extracellular location were included in the final evaluation list as it was anticipated that this would make them more accessible for detection in host serum.

The filtering process performed in this chapter selected for genes which showed an up-regulation in expression across the time course of infestation of ≥1.8 fold change and was characterised as being present in at least one of the selected biofluids for practical reasons of pen-side testing. The rationale of selecting a fold change in gene expression of ≥1.8 was to ensure the selected genes showed a detectable expression change, in anticipation that the protein transcript would increase to a significant degree also, giving higher opportunity for detection of the protein in serum. The rationale for selecting up-regulated genes only was that if the protein transcript was also up-regulated, the BM protein would be more easily detectable in serum in a diagnostic test during a field outbreak at an unknown time post-infestation. In addition, it was desirable that the potential BM circulated in serum at a low level in healthy animals as it was anticipated that there would be less variation in BM levels between animals pre-infestation, compared to a down-regulated protein which may be present at a higher level pre-infestation and therefore may show an increased level of variation between individuals at its "normal" physiological level. At the protein

level, the half life of the potential BM may also be an important factor. For example, if a protein has a long half life, a protein which is down-regulated during disease is likely to remain at measurable levels for a considerable time after disease onset, which is not desirable in a test aimed at early disease diagnosis.

Following this initial filtering based on mean animal responses, each gene was selected based on high quality microarray expression data, after which it was important to complete a re-assessment of the remaining genes to determine the individual animal responses. Examining individual animal responses allowed the filtering out of BMs which were not considered to be robust across all animals. This was particularly important as the experiment which provided the data for the bioinformatic analysis (Burgess et al., 2012a) contained 3 sheep with naturally resolving lesions and 3 with expanding lesions (Table 2.1). This may have led to the elimination of genes which were up-regulated only as lesion size increased if the results were examined solely as a mean across all animals. It was therefore critical to include an individual animal assessment of the ability of each gene to correlate with lesion size so that this factor could be taken into account. An example of the gene expression across individual animals at each time point for the highly ranked *COLEC11* was included in Figure 2.3. When this information is compared to the lesion size data in Table 2.1, it is evident that the expression of this transcript reflected lesion size development. For example, sheep 3 had a naturally resolving lesion and the gene expression profile was flat compared with sheep 5 which had an expanding lesion illustrated by the steep up-regulation of *COLEC11* for this animal. The ability of the gene expression to reflect lesion size development was important

as lesion size can be used as a measure of disease progression in sheep scab. Genes such as *OXT* and *COLEC11*, which demonstrated this ability, were likely to encode proteins which may reflect current disease status.

The magnitude of the gene expression response to disease was also an important factor to identify as larger magnitude responses, even at the transcript level, may provide a greater opportunity for the detection of the protein in serum, as many of the blood proteins are known to be present in very small quantities (Kulasingam et al., 2010). Human plasma proteins are present in a range of concentrations over 12 orders of magnitude and 99% of plasma protein mass consists of only 22 proteins (Anderson and Anderson, 2002) suggesting that many of the proteins, which may potentially be of interest, will be present in very small quantities, e.g. tumour necrosis factor alpha (TNF-α).

The final ranking of BMs also included an assessment of the overall change of gene expression level at each of the time points measured through the time course of the infestation. This was considered to be a major factor in BM selection as, to be detected in serum during a field outbreak at an unknown time post-infestation, it would be easier to detect and to interpret the test results if the protein was up-regulated across all time points during an active infestation. However, due to the limitations of experimental infestation of sheep with *P. ovis*, based on animal welfare considerations, it was only possible to measure changes for a 6 week period which may not fully reflect protein profiles in field outbreaks of sheep scab.

Due to the time and cost restraints of this project, the final selection of potential BM candidates retained for further evaluation depended on the availability of

commercially produced antibodies. At the time of study, antibodies were commercially available for the detection of eight of the remaining highly ranked proteins, as shown in Table 2.3 and these proteins were retained for further investigation. There were no ovine specific antibodies available for any of the highly ranked BMs at the time of testing. There is a lack of well-characterised tools and reagents which are required for veterinary immunology to further understand host/pathogen relationships (Entrican et al., 2009) which is currently being addressed by the Veterinary Immunology Committee Toolkit (Entrican and Lunney, 2012). It was decided therefore, due to the predicted conserved function of many of the immune response proteins across mammalian species, to use human specific antibodies where there was no other alternative. The proteins encoded by the highest ranked genes such as *ICAM3,* transient receptor potential cation channel, subfamily C, member 6 (*TRPC6)* and adenosine A3 receptor (*ADORA3)* had no suitable human specific antibodies available at the time of study. However, these genes may be revisited in the future as commercial antibodies become available and as such, this list represents a valuable resource for future investigations.

The eight proteins retained for further evaluation all showed functions relating to the immune system, particularly in response to inflammation. For example osteopontin (SPP) has been the subject of a large body of research due to its chemotactic properties in mammals, resulting in recruitment of cells such as macrophages and neutrophils to sites of inflammation such as those caused by infection, trauma or injury (Wang and Denhardt, 2008, Konno et al., 2011). The role of SPP in immune function is complex and recent research has concentrated on emerging roles in

wound inflammation, repair and fibrosis, through its function in Th1 cell mediated skin inflammation and in the Th2 regulation pathway in allergic dermatitis (Seier et al., 2010). Human keratinocytes can produce SPP under inflammatory conditions as shown by immuno-histochemical staining of skin biopsies from patients with psoriasis, an immune mediated inflammatory disease (Buback et al., 2009).

In relation to sheep scab, the pro-inflammatory response is likely to be initiated through the interaction of the mite antigens and the epidermal keratinocytes as *P. ovis* is a surface feeding mite (Watkins et al., 2009). The increase in the transcription of *SPP* during infestation with *P. ovis* may be explained if ovine keratinocytes, like human cells, produce SPP under inflammatory conditions, then the promotion of inflammation and resulting prolonged healing time and wound fibrosis would enhance the mites feeding capacity. This ability of the mite to use the host's innate inflammatory response for their own benefit has been suggested previously as the exudate, resulting from the keratinocyte response to mite allergens, becomes a major food source for the mites (Huntley et al., 2005).

In livestock research, SPP has previously been investigated as a potential early innate BM of mastitis in dairy cattle. *SPP* transcripts were shown to be up-regulated in the somatic cells of cows experimentally infected in the udder with *E. coli* and this correlated with the number of milk immune cells and susceptibility to mastitis, leading to an interesting possibility for selection of resistance to mastitis in dairy cattle using SPP as a selection BM (Alain et al., 2009).

Related roles in inflammatory disease can be attributed to all the other selected potential BMs. However, with the exception of C4BPB, results from the Western

blot analyses in this study showed either a lack of binding between the primary antibody and the BM being evaluated; or interaction of primary or secondary antibodies with ovine IgG in the sera samples. Serum contains large quantities of immunoglobulins which can interfere with the detection of proteins at these specified MWs (Signore and Reeder, 2012) for which a recognised method to minimise this is the use of immunoglobulin removal techniques, as applied in this study. This was a useful technique as it resulted in blots with less non-specific banding which were clearer to interpret, but it failed to remove all of the IgG present therefore interpretation of the blots remained a problem if the BM protein was of a similar MW to IgG, as for example was the case for COLEC11 and TNC at 25 kDa (MW of light chain IgG) and SLC7A11 at 50 kDa (MW of heavy chain IgG). In addition, a number of serum proteins have been shown to adhere strongly to immunoglobulins and therefore IgG removal may also remove potential BMs (Veenstra et al., 2005).

Another problem with the current study was the lack of commercially available ovine specific antibodies. All of the antibodies used were raised against the human version of the protein under investigation, except TNF where an antibody raised in mice against bovine TNF was used. Table 2.5 contains the comparable amino acid sequence alignments and illustrated a large range of percentage identities between the species compared. Using S100A9 as an example, the Western blot analysis failed to show distinct bands at any time point in the infestation study at the expected MW of approximately 16kDa but given the alignment of the human and bovine amino acid sequences it is possible that the two sequences showed insufficient identity (51%) for the antibody raised against the human recombinant protein to react

specifically with the ovine native protein. Previous work (Marchalonis et al., 2001) documented a linear correlation between degree of sequence identity and antigenic cross-reactivity; therefore it is possible that at 51% the identity between these sequences was too low to enable strong, specific antibody binding. However, a cut-off of 50% identity level between proteins has been used by the Protein Information Resource (PIR, Georgetown University Medical Centre) to denote that the proteins belong to the same family and are likely to have similar three dimensional structures. Taking this into consideration, the human and bovine sequence identities would have been marginal for the anticipation of structurally similar proteins and this may, in part, explain the lack of binding in the S100A9 immunoblots. As there was no available information from the manufacturers on the epitopes used in the production of these antibodies, it is difficult to assess their likelihood of cross-reacting.

The problems arising from the use of cross-species immunoblotting include variations in antigenic binding even in situations of high homology between the proteins under investigation (Hudgens et al., 2011). For example, unexpected differences in antibody binding affinity between species have recently been recognised in human cancer research, where pre-clinical models frequently used include mice, rats or monkeys as their protein identities, and therefore predicted cross-species antibody/antigen binding, to humans are high (Xin et al., 2012). However, recent results examining the binding capacity of the human monoclonal antibody against neuropilin-1, a membrane bound co-receptor for vascular endothelial growth factor in humans, rats, mice and monkeys, gave highly variable

binding affinities across these species which was not consistent with the predicted binding affinity (Xin et al., 2012).

A reason which is cited for weak antibody/antigen binding is species variation in post-translational modifications such as glycosylation (Pomes, 2010). Tertiary protein structure is important for the exposure of conformational epitopes which then affects antibody binding site availability and can account for lack of antibody binding (Pomes, 2010). However, in this study all the antibodies used were either raised against a recombinant or synthetic peptide which would be unlikely to have any glycosylation sites, so differences in glycosylation between species would be irrelevant in this instance.

It is evident that the highly ranked potential BMs evaluated in this chapter for which specific binding was not obtained using a cross species antibody, such as SPP, S100A8, S100A9, OXT and KCNQ1, should be re-examined when specific antibodies raised against the ovine or bovine proteins become available. The primary antibodies used to detect these proteins had not been tested against the respective ovine proteins, therefore in the absence of protein bands at the predicted MW, it is likely that the antibodies either did not recognise the protein, or the protein was not present in sufficient quantities to be detected. Identification of Western blot bands is achieved using positive controls, generally a recombinant protein, or by raising species specific antibodies. For this stage of screening, when multiple potential BMs were being evaluated, these techniques would have been impractical in terms of time and cost. Using mass spectrometry (MS) techniques to identify blood proteins present during infestation may have been an option although as previously

mentioned, the techniques of sera separation required for MS cannot as yet cope with the complexity of proteins in sera (Kulasingam et al., 2010). The numbers of proteins, however, obtained from separation by cation exchange liquid chromatography followed by mass spectrometry were increased by 3-5 fold in one study and including several low abundance serum proteins (ng/ml range) which could have been an alternative approach for the present study (Adkins et al., 2002).

The immunoblots from this preliminary evaluation indicated that C4BPB may have potential as a BM for sheep scab and, as such, warranted further investigation. Human C4BPB is a 28 kDa protein consisting of a unique beta chain which assembles with seven identical alpha chains (C4BPA) to form the major isoform of C4BP, a large (570 kDa) glycoprotein complex (Dahlback, 1983). The C4BP molecule, comprising the alpha and beta chains, is assembled in the endoplasmic reticulum after which it is secreted into the blood where its plasma level has been shown to increase four-fold during inflammatory disease in humans such as systemic lupus erythematosus (Barnum and Dahlback, 1990). As shown in Figure A2.2, C4BP is a regulator of the classical and lectin pathways of complement and as such is an important immune function protein. As the complement pathways are potentially destructive to the host, they are tightly regulated by several factors including C4BP (Blom et al., 2004). C4BPB, combined with C4BPA in its major isoform C4BP, can therefore be described as an inhibitor of the classical complement pathway and controls C4b-mediated reactions in three ways. Firstly it is a co-factor to factor 1 (F1), a serine protease which prevents the formation of the C3-convertase complex (C4bC2a) (Scharfstein et al., 1978). C4BP also prevents the C3-convertase assembly

by binding directly to C4b and finally, it accelerates the natural decay of the C3-convertase complex (Gigli et al., 1979).

At the time of study there was no sequence available for ovine *C4BPB*, but the evaluation of C4BPB as a potential BM for sheep scab by Western blotting using a monoclonal C4BPB antibody raised in mice against a human recombinant C4BPB protein, gave promising results. The band densities at the estimated MW of C4BPB correlated with disease progression showing increasing band intensities through the time course of infestation (Figure 2.5) and also correlated with lesion size (Figure 2.6). However, the immunoblot shown on Figure 2.5 illustrated a problem with using Western blot analysis to evaluate BMs, as multiple bands at various MWs can be identified. It is acknowledged that often proteins of different MWs, in addition to the expected MW, can be detected in Western blotting which can be due to post-translational modification of the target protein, splice variants, protein dimers or cross-reactivity between different proteins (Signore and Reeder, 2012).

To minimise the chances of the band of interest belonging to a different protein from the target, a commercially available human rC4BPB was used as a positive control, which confirmed the bands obtained in the immunoblots were likely to be C4BPB as shown in Figure 2.5. This recombinant protein also provided a standard against which the densities of the C4BPB bands at each time point across the course of infestation and lesion size could be quantified and compared (Figure 2.6). This showed that the band densities detected in the sera of two sheep experimentally infested with *P. ovis* correlated with lesion size development. This is an important factor in the search for a BM to indicate current disease status, as it suggests that

C4BPB may decrease to low circulating levels as the disease resolves, a finding which agrees with previous work on C4BPB where it was shown to track the severity of the APR in several human inflammatory diseases (Zadura et al., 2009, Ma et al., 2010).

However, further evaluation is required to clarify the potential of C4BPB as a BM for sheep scab. It is critical that it can be quantified across both experimental and field positive and negative sample sets and that it can be shown to increase significantly during early *P. ovis* infestation, returning to baseline levels rapidly post-treatment or with disease resolution. In order to further evaluate the potential of C4BPB as a BM of sheep scab in the next chapter, the ovine *C4BPB* gene is sequenced and a recombinant protein expressed, enabling an ovine specific anti-C4BPB antibody to be raised in rabbits. An ELISA is then developed to facilitate the cost effective screening of the many positive, negative and field samples required to further validate the potential of C4BPB as a BM for sheep scab.

2.5 Conclusions

Prior to this project, microarray analysis was used to examine gene expression in circulating leukocytes across a time course of infestation with *P. ovis* (Burgess et al., 2012a). The current project commenced with the bioinformatic filtering and ranking of differentially expressed genes from this study, to provide a list of potential BMs for sheep scab which fulfilled the criteria described in this chapter.

The final 178 genes, having passed the criteria applied and ranked according to BM potential, were selected for evaluation according to list ranking and on the availability of suitable commercial antibodies. This was essential to permit visualisation of the BM proteins by Western blotting in the sera of sheep infested with *P. ovis* compared to that of non-infested sheep sera. However, there were no ovine specific antibodies available for the selected proteins at the time of study which is a general issue with ruminant reagents, and the problems of using non-species specific antibodies to probe for the proteins being evaluated as potential BMs led to the conclusion that many of the highly ranked proteins obtained from the bioinformatic analysis should be re-examined as and when antibodies raised against the ovine (or bovine) proteins become commercially available.

However, results from the initial evaluation of C4BPB showed bands at the expected MW of 28 kDa which increased in density through the time course of infestation and with lesion size development. This suggested there was an increasing concentration of C4BPB in ovine sera and that this may correlate with the degree of disease progression. Band densities, and hence sera protein concentration, were also shown to decrease as the lesion of one experimentally infested sheep resolved naturally.

However, due to the semi-quantitative nature of this initial evaluation, and the small number of experimentally infested sheep involved, further evaluation is required to quantify C4BPB levels in non-infested and infested sheep (experimental and field samples) and post-treatment. This will be achieved by raising an ovine specific anti-C4BPB antibody, as described above, to allow development of an ELISA and is the subject of Chapter 3.

Chapter 3: Characterisation of C4BPB as a potential biomarker for sheep scab

3.1 Introduction

In the search for BMs which would indicate current disease status in sheep infested with *P. ovis*, complement 4 binding protein, beta chain (*C4BPB*) was one of the highest ranking genes examined in the bioinformatic analysis of potential BMs (Chapter 2) based on the following criteria: the *C4BPB* transcript level was highly up-regulated across the time course of *P. ovis* infestation; this up-regulated response occurred across all the time points examined (0, 1, 3 and 6 weeks post-infestation) with a "very good" time tracking score, a "good" lesion tracking score across individual animals and a high response magnitude (Chapter 2, Table 2.2) .

The results of the preliminary evaluation of C4BPB as a potential BM by Western blot analysis, using a 6 week time course of infestation pooled sera (n=6) (TC1) and a monoclonal antibody raised in mice against human C4BPB, confirmed that the density of bands obtained, at the predicted molecular weight of 28 kDa for human C4BPB, increased with disease progression (Chapter 2, Figure 2.5). Further to this the density of the bands and, accordingly, the concentration of C4BPB in serum, increased for one sheep experimentally infested with *P. ovis* (TC1) with an expanding lesion across a six week time course of infestation, whereas the band densities increased then decreased in a sheep with a naturally resolving lesion (Chapter 2, Figure 2.6). Although only based on two animals, this result indicated that C4BPB serum concentration may be an effective indicator of disease progression

and therefore was a potential BM indicating current disease status during *P. ovis* infestation.

C4BPB is an important immune function protein due to its role as an inhibitor in the complement cascade (Blom et al., 2004) but it also has been implicated in the activation of B cells through its interaction with CD40, a protein receptor required for the activation of antigen presenting cells (APCs) (Banchereau et al., 1994). C4BPB has also recently been investigated as a potential BM for several human inflammatory conditions. Along with SAA, serum C4BPB levels were significantly increased in patients with Takayasu arteritis, compared with unaffected controls (Ma et al., 2010). C4BPB sera concentration has also been shown to correlate positively with the severity of the APR in human patients with primary Sjögren's syndrome (pSS) and as such C4BPB has been identified as a possible marker of disease severity (Zadura et al., 2009). Through its binding with protein S, a vitamin K dependant plasma glycoprotein, C4BPB has been linked with susceptibility to venous thrombosis (VT) where recent work has provided evidence that genetic susceptibility for VT is found in the *C4BPB/C4BPA* locus (Buil et al., 2010). Although the exact mechanism has not been elucidated as yet, this locus to VT appears independent of the protein S regulation, opening a new research area focusing on the C4BP regulatory pathway (Buil et al., 2010).

Human C4BPB can also circulate bound to the vitamin-K dependant protein S, an anti-coagulant protein (Hessing, 1991). This association has been shown *in vitro* to enhance the interaction of C4BPB with neutrophils, which has been suggested as a mechanism by which neutrophils are protected from complement damage

(Furmaniak-Kazmierczak et al., 1993). There has recently been a rapid expansion in knowledge regarding complement evasion strategies by pathogens. Pathogens such as viruses, bacteria and parasites can bind C4BP, and other complement inhibitors such as factor H, to avoid clearance and destruction by the host's complement system (Blom et al., 2009). Complement is considered particularly important during the early stages of infection and is activated not only in blood, but on mucosal surfaces such as the bronchial and epithelial linings early in the immune response. Therefore the ability of pathogens to avoid killing by complement is an important determinant of their pathogenicity (Blom et al., 2009). For example, *Staphylococcus aureus* is the major cause of human skin and soft-tissue infections as well as invasive infections like post-operative wound infections and septic arthritis. It has been demonstrated that *S. aureus* M proteins (bacterial virulence factors) can bind C4BP from the host serum to the bacterial surface, reducing phagocytosis and suggesting a previously undescribed immune evasion strategy for this pathogen (Hair et al., 2012). Similar mechanisms for complement evasion have recently been described for *Yersinia pseudotuberculosis* (Ho et al., 2012), *Escherichia coli* (Tseng et al., 2012) and *Leptospira interrogans* (Domingos et al., 2012). Having described a similar evasion strategy in many pathogens, research is now being directed on finding vaccine candidates from bacterial proteins that interact with complement and in identifying methods of manipulating the complement system to develop novel therapeutic approaches (Blom et al., 2009).

As infestation with *P. ovis* results in the development of a rapid inflammatory response (Van den Broek et al., 2000), it is anticipated that one of the early host

responses to disease will be activation of the complement cascade and as this is potentially damaging to the host, inhibitors such as C4BPB are likely to be up-regulated (Blom et al., 2004). As such, C4BPB is a potential BM for sheep scab, due to its biological functions, its proposed use in tracking the severity of infection in other diseases and based on the results of Chapter 2 of this study.

To progress further with the evaluation of ovine C4BPB as a BM for current disease status in sheep scab, a quantitative approach was required involving the development of an ELISA test for ovine C4BPB. It is advantageous to use this approach compared with Western blotting techniques which, although useful in the preliminary stages of BM validation (Signore and Reeder, 2012), can only provide semi-quantitative values. In order to provide sufficient evidence of a protein's potential as a BM and to be able to incorporate it into a diagnostic test, quantitative measurements are required and, as such, assay development is a routine method of achieving BM validation (Brookes et al., 2010). In addition, ELISA development is also required due to the high number of sera samples, from both experimental and field positives and negatives, which need to be analysed in the validation of a potential BM (Kulasingam et al., 2010).

During the Western blot evaluation of C4BPB in Chapter 2, an antibody raised in mice immunized with full length human recombinant C4BPB (rC4BPB) was used to detect ovine C4BPB as there was no commercially-available antibody with specificity for ovine or bovine C4BPB. This antibody bound C4BPB in sheep serum in immunoblots and the cross-reactivity obtained may be a result of high levels of conservation between the sequences of some immune inflammatory proteins in

mammalian species (Aggarwal and Gurney, 2002). Comparisons between the bovine (as there was no ovine sequence available) and human C4BPB protein sequences showed a 49% amino acid identity (Chapter 2, Table 2.5) which is very close to the 50% identity level cut-off used by the Protein Information Resource (PIR, Georgetown University Medical Centre) to denote that proteins may belong to the same family, are likely to have similar three dimensional structures and hence cross-react. In addition, the cost of further BM validation using commercially-available antibodies alone would be prohibitive to screening the high quantity of samples required in the validation of a potential BM. Therefore the production of an ovine specific antibody was advantageous to the further evaluation of C4BPB as a potential BM.

The aims of this chapter were therefore to clone, sequence and characterise the ovine *C4BPB* gene; express a recombinant C4BPB protein based on this sequence; raise antibodies in rabbits specific to recombinant ovine C4BPB and to develop an ELISA allowing quantification of C4BPB in the sera of sheep prior to infestation with *P. ovis,* across the time course of infestation and post- treatment.

3.2 Materials and methods

3.2.1 *C4BPB* cloning and sequencing

The methods described in this section involved gene amplification using degenerate primers, followed by Rapid Amplification of cDNA ends (RACE) to generate full-length sequence of the transcript encoding ovine *C4BPB.*

3.2.1.1 Partial gene sequencing

3.2.1.1.1 RNA extraction and cDNA synthesis

Complementary DNA (cDNA) to be used as a template in the amplification of the ovine *C4BPB* gene, was prepared from RNA derived from ovine circulating leukocytes of sheep 6 weeks post-infestation with *P. ovis* (see chapter 2, section 2.2.3.6 (TC1) for sample description). The RNA used was extracted from leukocytes by filtering whole blood (Burgess et al., 2012a) as described in that study. cDNA was synthesised using SuperScript™ II Reverse Transcriptase (Invitrogen), following the manufacturer's protocol, with the following reaction: Oligo(dT)$_{23}$ primer (Sigma) at 500µg/ml; dNTPs (10mM) (Invitrogen) and RNA template (480ng) to a total volume of 12µl were incubated at 65°C for 5 mins. RNaseOUT (Invitrogen) was added at 40 units/µl to inhibit the action of RNases on the template RNA, before incubating at 42°C for 2 mins. Superscript II (Invitrogen) (200 units) was added prior to a final incubation at 42°C for 15 min and 70°C for 15 min.

3.2.1.2.2 Amplification of gene fragment by PCR

Nucleotide sequences, available from the National Centre for Biotechnology Information (NCBI), representing porcine *C4BPB* (Accession number: AK 346434), bovine (NM 174253) and human (NG 029386) were aligned using ClustalW2

[European Bioinformatics Institute, (EBI)]. Areas of high sequence similarity were identified as possible oligonucleotide primer binding sites to enable the design of degenerate primers (Figure 3.1).

```
AK 346434 Pig        AGTGCAGTGAGCATTACATCCTCAAGGGCAGCAATTGGAGCCGGTGCCGAGACGACCACA 303
NG 029386 Human      TGTGCAATGACCACTACATCCTCAAGGGCAGCAATCGGAGCCAGTGTCTAGAGGACCACA 373
NM 174253 Bovine     AATGCAATGAGCATTGCATCTTTAAGGGCAGCAATTGGAGCCAGTGTCGAGAAAACCACA 480
                       **** *** ** * **** * ************ ****** *** * ***  ******

AK 346434 Pig        CCTGGGTGCCTCCCTTTCCCATCTGTAAAAGCAAAGACTGTGGCCCTCCTGAGAATCCAA 363
NG 029386 Human      CCTGGGCACCTCCCTTTCCCATCTGCAAAAGTAGGGACTGTGACCCTCCTGGGAATCCAG 433
NM 174253 Bovine     CCCGGGTGACTCACTCTCCTGTGAGCAAAAGCAGAGACTGTGGCCCTCCTGAGACTCCAA 540
                     ** ***   *** ** ***  *  * ***** *  ******* ******** ** ****

AK 346434 Pig        TTCATGGCTATTTTGAAGGAATCGATTTCAACTCAGGGTCTACCATTACTTATTACTGTA 423
NG 029386 Human      TTCATGGCTATTTTGAAGGAAATAACTTCACCTTAGGATCCACCATTAGTTATTACTGTG 493
NM 174253 Bovine     CTCATGGCTATTTTGAAGGAAGAGATTTCAAGTCAGGATCTACCATCACTTATTACTGTC 600
                      ********************   * ****  * *** ** ***** * **********

AK 346434 Pig        AAGCGAGGTACCGCCTGGTGGGCACACAGCACCAACAGTGCATTGATGGGGAGTGGAGTG 483
NG 029386 Human      AAGACAGGTACTACTTAGTGGGCGTGCAGGAGCAGCAATGCGTTGATGGGGAGTGGAGCA 553
NM 174253 Bovine     AAGCAAGGTACCGCCTGGTGGGCACACAGCACCAACAGTGCATTGATGGGGAGTGGACCA 660
                     ***  ******  * * ******   *** * ** ** *** ***************

AK 346434 Pig        GTGCAATTCCAGTCTGTGAGCTGATCCCAGAAGCTCCCAAGGCAGCTTTGCAGATTGAGT 543
NG 029386 Human      GTGCACTTCCAGTCTGCAAGTTGATCCAGGAAGCTCCCAAACCAG------------AGT 601
NM 174253 Bovine     GTGCCCCTCCCATCTGTGAGTTGATCCAAGAAGCTCCCAAACCAGCCG---AATTAGAGT 717
                     ****   ***  ****  ** ******  ***********  ***           ***

AK 346434 Pig        TTGAGAAGGCCCTTCTTGCCTTTCAGGAAAGTGAGGAACTGTGCAAAGCCACTGAGAACT 603
NG 029386 Human      GTGAGAAGGCACTTCTTGCCTTTCAGGAGAGTAAGAACCTCTGCGAAGCCATGGAGAACT 661
NM 74253 Bovine      TGGAGAAGGCATTTCTTGCCTTTCAGGAGAGTAAGGAACTTTGCAAAGCTATAAAGAAAT 777
                       ********  *************** *** ** * ** *** **** *   **** *
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Figure 3.1: Aligned porcine, human and bovine *C4BPB* sequences with high homology sequence areas for degenerate primer design, highlighted in red. Only partial gene sequences are shown, representing the portion used for primer design. Actual primer sequences used are presented in Table A3.1 *Marks the position of identical nucleotide bases in the sequences for all three species.

Using these degenerate primers, in combination with cDNA prepared as described above (section 3.2.1.1.1), a portion of the ovine *C4BPB* gene was amplified by polymerase chain reaction (PCR) with the following reaction: primers at a final dilution of 2µM; template cDNA (106.5µg); dNTPs (Roche) at 10mM and 5 units

Taq polymerase (Roche) combined in a final volume of 30μl. PCR amplification was performed using a GeneAmp PCR System 2700 (Applied Biosystems). Cycling conditions were: 94°C for 5 min; 30 cycles of 94°C for 30s, 57°C for 30s, 72°C for 1 min; 72°C for 10 min final extension. PCR products were separated by electrophoresis on a 1% agarose gel incorporating GelRed stain (as described in A3.1.2) and the PCR amplicon was purified using a QIAquick PCR Purification Kit (Qiagen) following the manufacturer's protocol.

3.2.1.2 Cloning PCR product into the pGEM-T plasmid

The purified PCR product was ligated into the cloning vector pGEM-T (Promega) using T4 DNA ligase (Promega) transformed by heat shock (42°C for 40s) into *Escherichia coli* JM109 High Efficiency Competent Cells (Promega) and cultured in Luria Bertani (LB) medium prior to selection on LB agar containing ampicillin (100 μg/ml), 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as described in A3.1.3. Colonies with plasmids containing the cloned partial gene sequence for *C4BPB* were selected using the principle of alpha complementation, isolated and plasmid DNA extracted using a Wizard® Plus SV Miniprep kit (Promega) following overnight culture in LB medium containing ampicillin (100 μg/ml). The purified plasmid DNA was sequenced by Source Bioscience (Life Sciences, UK) with Sanger sequencing using T7 oligonucleotide primers. Sequencing results were aligned in the CAP3 Sequence Assembly Programme [Pole Bioinformatique Lyonnaise (PBIL)] and the contiguous sequence obtained used in homology searches using the NCBI Basic Local Alignment Search Tool (BLASTx) programme.

3.2.1.3 Generation of full-length sequence for ovine *C4BPB* using Rapid Amplification of cDNA Ends (RACE)

To extend the partial gene sequence for ovine *C4BPB* and obtain a full coding sequence (CDS), RACE was employed using primers designed to allow conventional PCR for 5' RACE and touchdown PCR for 3' RACE. Using the primer design protocol in the SMARTER™ RACE cDNA Amplification User Manual (PT4096-1, Clontech) and the software Primer 3 Output (SourceForge), gene specific primers (GSPs) for 5' and 3' RACE were designed, based on the partial *C4BPB* gene sequence. Nested primers were designed in the same way but selected from sequences positioned between the GSPs (see Figure 3.2).

```
1   K  G  S  N  W  S  Q  C  R  E  N  H  I  W  V  T  H  S  P  V
1   TTAAGGGCAGCAATTGGAGCCAGTGCCGAGAAAACCACATCTGGGTGACTCACTCTCCTG
1            10         20         30         40         50
21  C  K  S  R  D  C  G  P  P  E  T  P  T  H  G  Y  F  E  G  R
61  TCTGCAAAAGCAGAGACTGTGGCCCTCCTGAGACTCCAACTCATGGCTATTTTGAAGGAA
61           70         80         90         100        110
41  D  F  K  S  G  S  T  I  T  Y  Y  C  E  A  R  Y  H  L  V  G
121 GAGATTTTAAGTCAGGATCTACCATCACTTATTACTGTGAAGCAAGGTACCACCTGGTGG
121          130        140        150        160        170
61  T  Q  H  Q  Q  C  I  D  G  E  W  T  S  A  P  P  I  C  E  L
181 GCACACAGCACCAACAGTGTATTGATGGGGAGTGGACCAGTGCCCCTCCCATCTGTGAGT
181          190        200        210        220        230
81  I  Q  E  A  P  K  P  A  E  L  A  L  E  K  A  L  L  A  F  Q
241 TGATCCAAGAAGCTCCCAAACCAGCCGAGTTAGCGTTGGAGAAGGCCCTTCTTGCCTTTCA
241          250        260        270        280        290
```

Figure 3.2: Nucleotide sequence and translation map (Sequence Manipulation Suite, Bioinformatics Organisation) of the *C4BPB* gene fragment generated using the degenerate primers showing the position of the gene specific primer (GSP) (yellow background) and nested primers (internal primers with blue background for the forward primer and green for reverse) used for 5' RACE. Primer names and sequences are described in Table A3.1.

Primers were obtained from MWG Eurofins. RACE- ready cDNA was generated from total RNA extracted from sheep leukocytes (see section 3.2.1.1.1) using the SMARTER™ RACE cDNA Amplification kit (Clontech, US) and following the manufacturer's protocol. To obtain a 5' RACE product, PCR amplification was performed using 1 unit SMARTIIA oligonucleotide (Clontech) in conjunction with a GSP (see Table A3.1 and Figure 3.2) at a final concentration of 10µM, 1 unit 50X Advantage 2 polymerase, Advantage 2 dNTPs (10µM) and 5'RACE Ready cDNA (244ng). Reaction parameters were as follows: 20 cycles at 95°C for 30s; 68°C for 30s; 72°C for 3 mins. To further amplify the 5' RACE reaction product, it was purified using a QIAquick PCR Purification Kit (28704, Qiagen) and 3µl used as template in a PCR amplification reaction with the "Nested reverse RACE primer"(10µM) as shown in Table A3.1, nested universal primer (NUP) (Clontech), Advantage 2 polymerase and Advantage 2 dNTPs (Clontech) as per manufacturer's protocol, with reaction parameters as follows: 20 cycles: 95°C for 30s; 56°C for 30s; 72°C for 3 mins.

The purified PCR product was ligated into the cloning vector pGEM-T (Promega) using T4 DNA ligase (Promega), transformed by heat shock (42°C for 40s) into *E. coli* JM109 High Efficiency Competent Cells (Promega) and plasmids containing the 5' RACE product were amplified, purified and sequenced using M13 uni (-21) primers.

Sequence results were aligned using CAP3 (PBIL) and translated in the Sequence Manipulation Suite (Version 2, Bioinformatics Organisation). Homology searches were performed using the NCBI BLASTp program and the translated sequence was

aligned with the bovine C4BPB protein sequence (accession number NM 174253) in ClustalW2 (EBI) to determine sequence homology.

The remainder of the ovine *C4BPB* gene was amplified using a GSP for 3' RACE (see Table A3.1) based on the 5' RACE product sequence, and used at a final concentration of 10μM in a touchdown PCR reaction with 1 unit SMARTIIA oligonucleotide (Clontech), 3' RACE-ready DNA (244ng), 1 unit 50X Advantage 2 polymerase and 10μM Advantage 2 dNTPs (Clontech). The reaction parameters were as follows: 5 cycles at 94°C for 30s, 72°C for 3 min; 5 cycles at 94°C for 30s, 70°C for 30s, 72°C for 3 min; 25 cycles at 94°C for 30s, 68°C for 30s and 72°C for 3 min. The purified PCR product (3μl) was ligated into the cloning vector pGEM-T (Promega) using T4 DNA ligase (Promega) as per manufacturer's protocol, transformed by heat shock (42°C for 40s) into *E. coli*, JM109 High Efficiency Competent Cells (Promega) and plasmids containing the 3' RACE product were sequenced using M13 uni(-21) and M13 rev(-29) primers by MWG Eurofins.

3.2.2 Expression of recombinant ovine C4BPB

Two different strategies were employed to express recombinant ovine C4BPB protein in bacteria. In the first, an Advantage InFusion HD Cloning kit (Clontech, US) was used to subclone the full-length CDS into the expression vector pET22b(+) (Novagen). In the second, the CDS was subcloned into pET SUMO (Invitrogen) vector using TA cloning.

3.2.2.1 Expression using pET22b(+)

Primers were designed using the full CDS of ovine *C4BPB* derived as described above and modified at their 5' termini with 15 nucleotide bases homologous to the insertion site of the multiple cloning site (MCS) of the vector. The InFusion forward primer (highlighted in green in Figure 3.3) was not designed to start at the initiating codon but 52 bases into the sequence, because the sequence between the initiation codon and base 52 encoded a signal peptide (highlighted in red in Figure 3.3), as established by the SignalP package (4.0 Server). PCR was performed using these primers at a final concentration of 10μM, with 108ng pGEM-T plasmid containing the full length ovine *C4BPB* CDS as template, 10mM Advantage 2 dNTPs and 1 unit 50X Advantage 2 Polymerase (Clontech) with reaction conditions as follows: 94°C for 5 mins; 30 cycles at 94°C for 30s, 55°C for 30s, 72°C for 1 min and a final extension at 72°C for 10 mins. The pET22b(+) vector (Novagen) was linearised using 2 units of the restriction enzyme *Xho*1 (Roche) in a 2hr incubation at 37°C and linearised vector product was purified using a QIAquick PCR Purification Kit (Qiagen) following the manufacturer's protocol.

For cloning, the PCR product obtained using the InFusion primers described above, was used in a 10:1 molar ratio with the digested plasmid and incubated with 5X InFusion HD Enzyme Premix, at 37°C for 15 min then 50°C for 15 min. The plasmids, with integrated ovine *C4BPB* CDS were then transformed, by heat shock into *E. coli*, JM109 High Efficiency Competent Cells (Promega) and cultured in LB medium prior to selection on LB agar plates containing ampicillin (100 μg/ml).

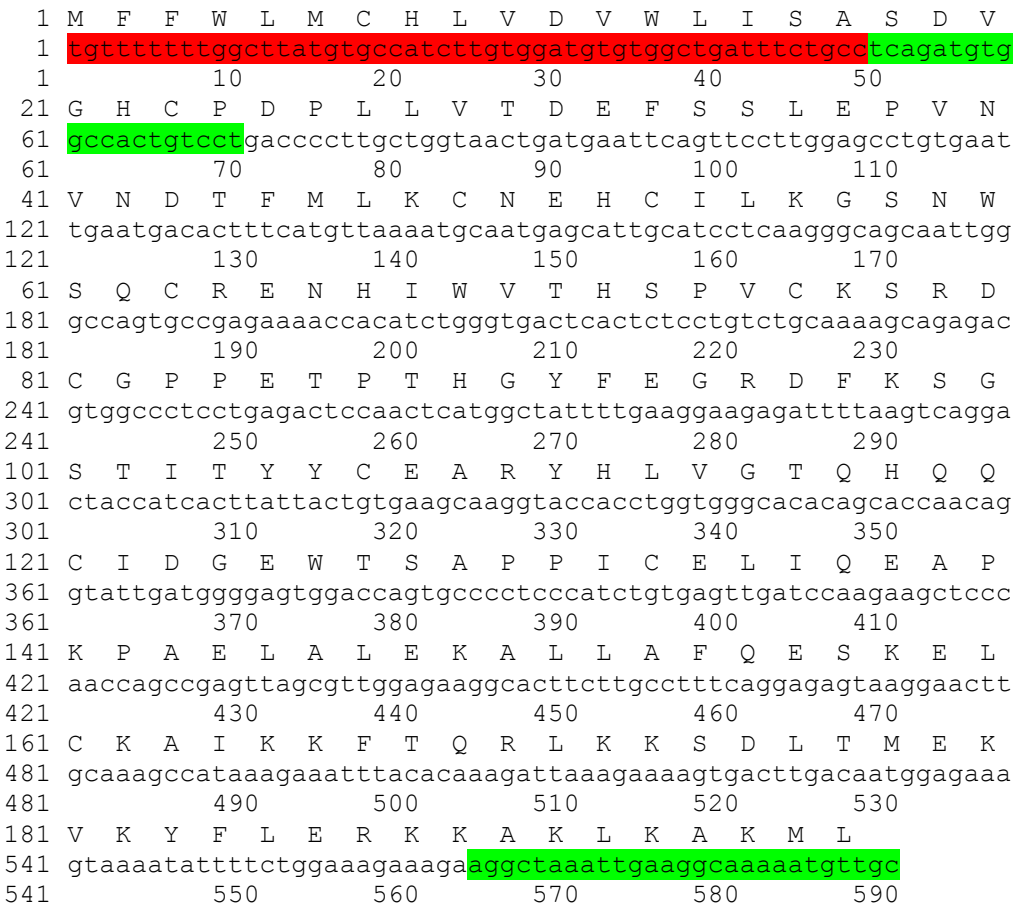


Figure 3.3: Translation map (Sequence Manipulation Suite, Bioinformatics Organisation) of full CDS of ovine *C4BPB* with nucleotide bases used in primer design highlighted in green. Full primer sequences used are included in Table A3.1 and are named "InFusion forward" and "InFusion reverse" primers. Signal peptide highlighted in red.

The purified plasmids containing the *C4BPB* CDS were sequenced using T7 primers prior to transformation into BL21 codon plus (DE3) *E. coli* cells (Agilent Technologies) by heat shock (42°C for 40s) and cultured on LB agar containing ampicillin (100mg/ml) and chloramphenicol (50mg/ml). Colonies containing the *C4BPB*-encoding plasmids were isolated and cultured overnight in 10ml LB medium

containing both antibiotics as described above and 750µl of this overnight culture were used to seed 50ml of LB medium, followed by incubation with shaking at 200rpm at 37°C until the optical density at 600 nm (OD_{600}) was 0.6. To obtain an "uninduced" sample, 1ml of the culture was removed before IPTG was added to the flask to a final concentration of 1mM and incubated at 37ºC for 3 hours, after which 0.6ml was removed ("induced"). Both uninduced and induced fractions were centrifuged for 10 mins at 19000*g* and the pellets, containing the *E. coli* cells with C4BPB-encoding plasmids, were retained at -80°C.

The pellets (uninduced and induced) were re-suspended in 100µl binding buffer, (A3.1.4) with 20% Triton-X 100 (Sigma-Aldrich) centrifuged at 22000*g* for 10 mins and the supernatant retained (soluble phase), with the pellets being re-suspended in 100µl binding buffer containing 8M urea (Appendix A3.1.5) (insoluble phase). Polyacrylamide gel electrophoresis (SDS-PAGE) (NuPage BisTris 4-12%, Invitrogen) was run using a total volume of 20µl of each sample (i.e. uninduced soluble, induced soluble, uninduced insoluble and induced soluble) after samples had been incubated with 5µl 4X NuPage LDS sample buffer (Invitrogen) and 2µl NuPage sample reducing agent (Invitrogen) at 70°C for 10 mins. Electrophoresis was performed at 250V for 35 minutes, before Coomassie staining using SimplyBlue Safestain (Invitrogen).

3.2.2.2 Expression using pET SUMO

The ovine *C4BPB* CDS sequence was cloned into an alternative expression vector, pET SUMO, using the Champion pET SUMO Protein Expression System (Invitrogen). The Advantage PCR Kit (Clontech) was used to amplify the CDS

(minus signal peptide) for ovine *C4BPB*, with the following parameters: 108ng pGEM-T plasmid containing the *C4BPB* CDS as template; pET SUMO forward and reverse primers 10μM (See Table A3.1), Advantage dNTPs 50mM; 1 unit 50X Advantage 2 polymerase; cycling parameters were as follows: 94°C for 5mins; 30 cycles at 94°C for 30s, 58°C for 30s, 72°C for 1 min and a final extension at 72°C for 10 min.

The resulting PCR product was purified using a QIAquick PCR Purification Kit (Qiagen) and, following ligation of the vector and plasmid (1:1 ratio) with 4 units T4 DNA Ligase (Invitrogen), transformation was by heat shock (42°C for 30s) into One Shot Mach1-T1 *E. coli* competent cells (Invitrogen) and incubation on LB agar containing 50μg/ml kanamycin. Colonies with plasmids containing the *C4BPB* gene sequence (minus signal peptide) were isolated; plasmids extracted using Wizard® Plus SV Minipreps (Promega) following the manufacturer's protocol and sequenced using T7 primers. Following confirmation of successful cloning, plasmid DNA was transformed into BL21 codon plus (DE3) *E. coli* cells (Agilent Technologies) and protein expression and analysis performed as described in section 3.2.2.1 above.

3.2.2.2.1 Matrix-assisted laser desorption ionization (MALDI)

To confirm the identity of the expressed recombinant protein, the band of interest (ca. 40kDa) in the induced, insoluble fraction lane was excised from a SDS-PAGE gel of the induced bacterial expression products and mass spectrometry (MS) using MALDI - time of flight (ToF) – ToF was performed at the MRI Proteomics facility. Briefly, proteins were destained and reductively alkylated using DTT and iodoacetamide. Gel pieces were digested overnight with porcine trypsin (Promega) at

37^{o}C. Digests were analysed on a Bruker Ultraflex II MALDI-ToF-ToF mass spectrometer (Bruker Daltonics), scanning the 600 to 5000 dalton region in reflectron mode producing monoisotopic resolution. The spectra generated were mass calibrated using known standards and masses obtained were searched using primary sequence databases (Mascot, Matrix Science) (K. McLean, Pers. Comm.).

3.2.2.2.2 Recombinant Protein purification

Pellets containing the induced, insoluble fraction of a 50ml culture were re-suspended in 1ml binding buffer containing 8M urea as described in A3.1.5. As cloning into pET vectors adds an N-terminal (pET SUMO) or C-terminal (pET22b(+) polyhistidine (6 x His) fusion tag to the recombinant protein, a 1ml HisTrapFF (GE Healthcare) column was used for protein purification by nickel-affinity chromatography using the manufacturer's protocol. Purified rC4BPB was eluted using solutions of increasing imidazole concentration in binding buffer at pH 7.4 as described in A3.1.6, and dialysed overnight against dialysis buffer (described in A3.1.7) prior to protein quantification by Bicinchoninic acid (BCA) assay using bovine serum albumin (BSA) standards (Pierce) as per manufacturer's protocol.

3.2.3 Anti-ovine C4BPB antibody production

Blood samples were taken, by venous extraction, from two 12 week old New Zealand White rabbits prior to immunisation by injection with 52.8µg recombinant C4BPB (rC4BPB) in 25µg Quil A adjuvant (Guinness Products). Immunisation was performed three times at three week intervals. Blood samples were taken two weeks after the second immunisation and both rabbits were culled for the terminal bleed two weeks after the final immunisation. This work was carried out at MRI under the

guidelines of the MRI experimental ethics committee (Experiment E38/11) and according to Home Office legislation.

To test the immune response of the rabbits following immunisation with rC4BPB, an SDS-PAGE gel [NuPage BisTris 4-12% (Invitrogen) using buffer described in A2.1.1] was performed using 13.74µg ovine rC4BPB incubated with 2.5µl 4X NuPage LDS sample buffer (Invitrogen) and 1µl NuPage sample reducing agent (Invitrogen) at 70°C for 10 mins and 10µl added to the gel wells. After electroblotting onto nitrocellulose membrane, the blot was probed with rabbit sera (obtained 2 weeks post second immunisation) from each immunised rabbit, at a dilution of 1:400. Sera from the rabbits pre-immunisation were used as negative controls. The secondary antibody used was swine anti-rabbit IgG HRP conjugate (Dako) at a 1:2000 dilution and the blot was visualised by staining with DAB (3, 3 – Diaminobenzidine) (Sigma Aldrich) according to the manufacturer's instructions.

To assess binding of immunoglobulins in the sera from the immunised rabbits to native ovine C4BPB, SDS-PAGE gel electrophoresis was used to separate 6 week post-infestation pooled sheep sera (1:10 dilution of sera from TC 1 experimental *P. ovis* infestation study as described in Chapter 2, section 2.2.3.6). Following this separation of the serum proteins by electrophoresis, they were electroblotted onto nitrocellulose membranes. This blot was then probed with sera (at a dilution of 1:100) obtained at terminal bleed (2 weeks after 3rd immunisation) from rabbits immunised with rC4BPB. Serum from the same rabbits, prior to immunisation, was used as a negative control. A positive control using 21.14 µg ovine rC4BPB was included. As a secondary antibody, swine anti-rabbit IgG HRP conjugate (Dako) was

used at a dilution of 1:2000 and visualised by using ECL Plus Western Blotting Detection Reagent (GE Healthcare) followed by X-ray plate development and visualisation on an ImageQuant LAS4000 (GE Healthcare) camera.

3.2.4 Development of a C4BPB ELISA

The reagents developed in sections 3.2.2.2 – 3.2.3 were combined for use in an ELISA to detect C4BPB in sheep sera: The work flow for the development of an ELISA to enable detection of ovine C4BPB in the sera of infested sheep is described in Figure 3.4. As the improved sandwich ELISA using a biotinylated conjugate yielded the most promising results, the detailed methodology for this ELISA is included in A3.1.10. All optical densities were read at 450nm (OD_{450}) using a 630nm filter as reference, on a Sunrise™ 96-well plate absorbance reader (Tecan).

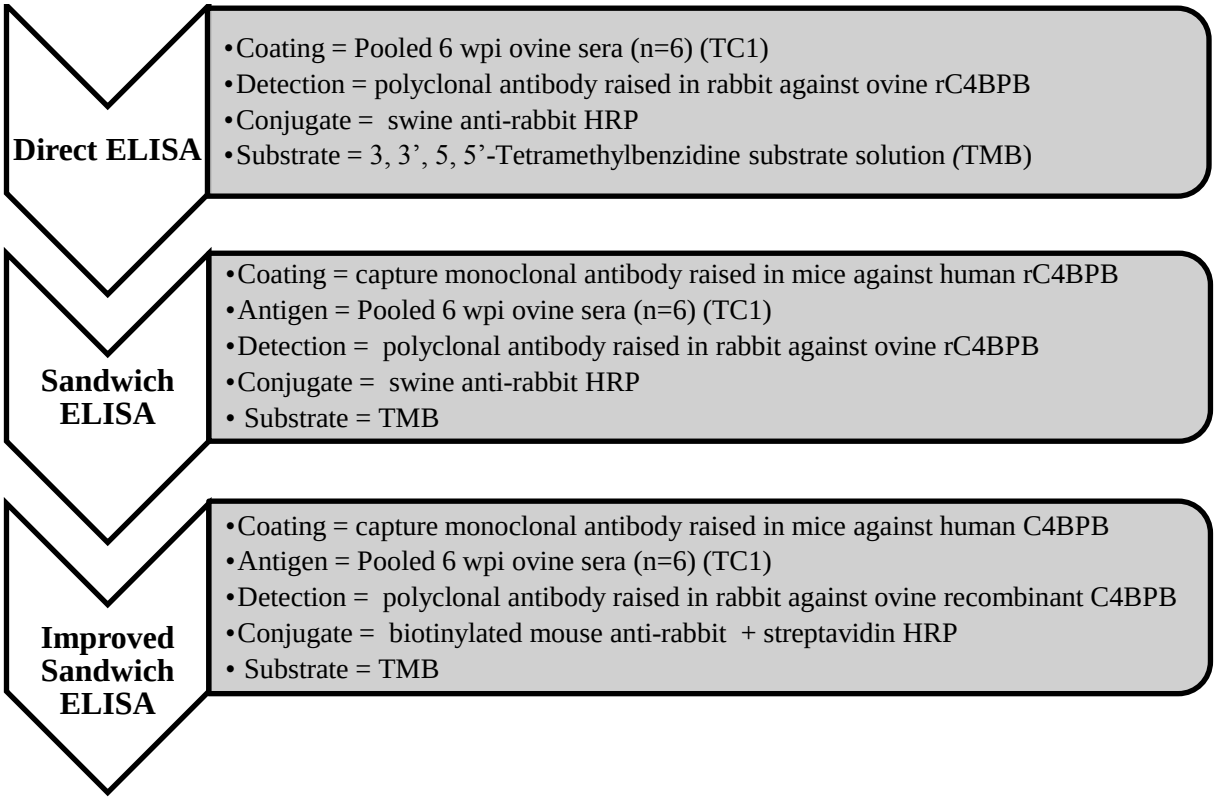


Figure 3.4: Work flow for ELISA development including the main steps and reagents used from the initial direct assay to the improved sandwich ELISA. Polyclonal C4BPB from rabbits immunised with ovine rC4BPB (as described in section 3.2.3) used at 1:100 dilution; mC4BPB raised in mice against human rC4BPB (Novus Biologicals) used at 1:100; swine anti-rabbit HRP IgG (Dako) used at 1: 2000 dilution; all ovine sera was diluted 1:10; biotinylated mouse anti-rabbit IgG (Abcam) used at a 1:100 dilution; Streptavidin HRP (Sigma) diluted 1:1000.

3.2.5 Measurement of C4BPB in sheep sera by ELISA

The optimised sandwich ELISA was tested using pooled sera from *P. ovis* infested sheep (n = 12) in an experimental primary infestation and treatment trial (TCT1 as described in A3.1.11). This testing was comprised of two components:

1) An initial comparison of ELISA OD_{450} between sera from sheep which had not been previously exposed to *P. ovis* (controls) and sera from the same sheep after a six week *P. ovis* infestation period (infested) was performed using pooled sera from 12 animals obtained from each of the groups in the experimental trial described in A3.1.11.

2) ELISAs were then performed using pooled sera from 12 animals from each sampling point of the trial described in A3.1.11, across a time course including pre-infestation, weekly samples from a 6 week infestation and sera taken from animals twice-weekly for 3 weeks post-treatment.

3.2.5.1 Statistical analyses

To analyse the difference in OD_{450} in the ELISAs comparing "control" sera (i.e. sera obtained pre-infestation with *P. ovis*) with "infested" sera (sera obtained 6 weeks post-infestation with *P. ovis*), a Student's paired t-test was used (GraphPad Prism, version 5.05, GraphPad Software Inc.).

In the analyses of the time course ELISA OD_{450}, the statistics used were repeated measures one-way ANOVA with Tukey's post-hoc test as calculated by GraphPad Prism (Version 5.05, GraphPad Software Inc.).

3.3 Results

3.3.1 Ovine C4BPB sequencing

3.3.1.1 Amplification of ovine C4BPB gene fragment by PCR

As a result of PCR amplification, using the degenerate primers described in section 3.2.1.2.2, a single amplicon was obtained of approximately 300bp. The results from sequencing and alignment using CAP3 (PBIL) confirmed that the sequences of 4 individual plasmids containing the cloned 301bp amplicon were identical. Homology searching using BLASTx (NCBI) confirmed that the contig obtained was a fragment of the gene representing *C4BPB* and that the translated amino acid sequence displayed 99% homology to that of the corresponding fragment of bovine *C4BPB* [accession number NM 174253 (EBI)]. The nucleotide sequence length obtained was 301 base pairs (bp), which when compared to the published bovine *C4BPB* sequence (594 bp), equated to approximately half of the putative CDS.

3.3.1.2 Generation of full-length sequence for ovine *C4BPB* using Rapid Amplification of cDNA Ends (RACE)

5’ RACE was successful in generating PCR products of 500+ bp in length (Figure 3.5). Cloning and sequencing of these products produced a consensus sequence representing the 5' end of the ovine *C4BPB* sequence. Following the successful identification of the 5’ region of the ovine *C4BPB* sequence, 3’ RACE was performed and the results of the initial 3’ amplification are shown in Figure 3.6.

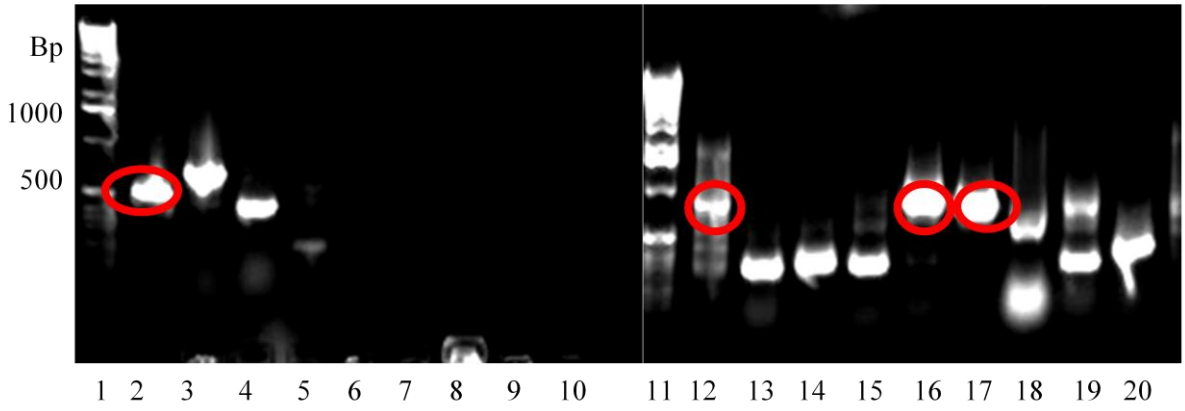


Figure 3.5: Gel image of the colony PCR of the cloned products of 5’ RACE indicating four plasmids (circled), containing longer inserts, which were subsequently sequenced.

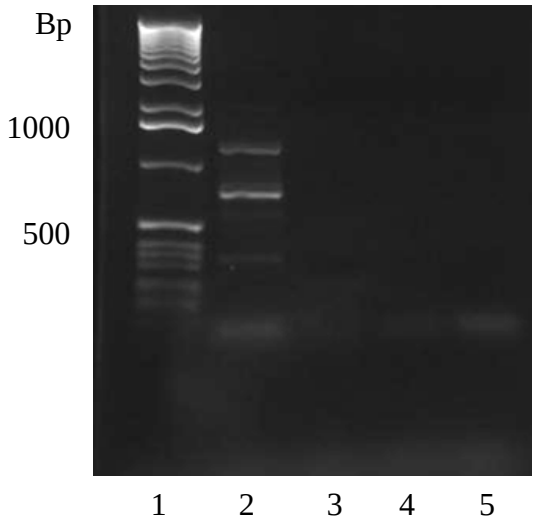


Figure 3.6: Gel image of the PCR results of 3' RACE using primers designed on the 5' RACE sequence. Lane 1 = Molecular weight marker X (Roche); Lane 2 = PCR product from 3’ RACE ready cDNA and GSP for 3’ RACE; Lane 3 = single primer control (GSP for 3’ RACE only); Lane 4 = single primer control (UPM only); Lane 5 = -ve control (no template).

In lane 2 there was a band evident at ca. 700bp and a weaker band at ca. 1200bp. By analogy to the bovine *C4BPB* nucleotide sequence, the lower molecular mass band was most likely to represent the ovine *C4BPB* and therefore this band was gel-purified, cloned and sequenced. There was 95% amino acid homology between ovine *C4BPB1* and the published sequence for bovine *C4BPB* and, as such all further work focused on ovine *C4BPB1* (Figure 3.7).

```
OvineC4BPB1      MFFWLMCHLVDVWLISASD----------------------------VGHCPDPLLVTDE 32
OvineC4BPB2      MFFWLMCHLVDVWLISASDGCGYSWTWENTRSLNTRSQAKRHYEGDSMGRCPDPLLVTDE 60
BovineC4BPB      MFFWLMCYLVDVWLISASD----------------------------VGHCPDPLLVTDE 32
                 *******:***********                             :*:**********

OvineC4BPB1      FSSLEPVNVNDTFMLKCNEHCILKGSNWSQCRENHIWVTHSPVCKSRDCGPPETPTHGYF 92
OvineC4BPB2      FSSLEPVNVNDTFMLKCNEHCILKGSNWSQCRENHIWVTHSPVCKSRDCGPPETPTHGYF 120
BovineC4BPB      FSSLEPVNVNDTFMFKCNEHCIFKGSNWSQCRENHTRVTHSPVSKSRDCGPPETPTHGYF 92
                 **************:*******:************  ******.****************

OvineC4BPB1      EGRDFKSGSTITYYCEARYHLVGTQHQQCIDGEWTSAPPICELIQEAPKPAELALEKALL 152
OvineC4BPB2      EGRDFKSGSTITYYCEARYHLVGTQHQQCIDGEWTSAPPICELIQEAPKPAELALEKALL 180
BovineC4BPB      EGRDFKSGSTITYYCEARYRLVGTQHQQCIDGEWTSAPPICELIQEAPKPAELELEKAFL 152
                 ******************:******************************** ****:*

OvineC4BPB1      AFQESKELCKAIKKFTQRLKKSDLTMEKVKYFLERKKAKLKAKML  197
OvineC4BPB2      AFQESKELCKAIKKFTQRLKKSDLTMEKVKYFLERKKAKLKAKML  224
BovineC4BPB      AFQESKELCKAIKKFTQRLKKSDLTMEKVKYSLERKKAKLKAKMLL 198
                 ****************************** *************
```

Figure 3.7: Clustal alignment of two contigs (*C4BPB1* and *C4BPB2*) derived from PCR amplification of the ovine *C4BPB* gene with the published bovine *C4BPB* sequence (accession number NM 174253) (Uniprot, EBI). Asterisks represent identity, colons represent high similarity, full stops represent low similarity and spaces represent no similarity.

3.3.2 Expression of recombinant C4BPB

3.3.2.1 Expression using pET22b(+)

Following InFusion cloning of the ovine *C4BPB* CDS into pET22b(+), a colony PCR of the resulting transformation products was performed using pET22b forward and reverse primers. From this PCR, a single colony was identified which included the pET22b(+) vector plus ovine *C4BPB* CDS insert as shown in Figure 3.8. Sequencing of the plasmid derived from this clone confirmed that the full *C4BPB* sequence (minus signal peptide) had been successfully cloned into the pET22b(+) vector in frame.

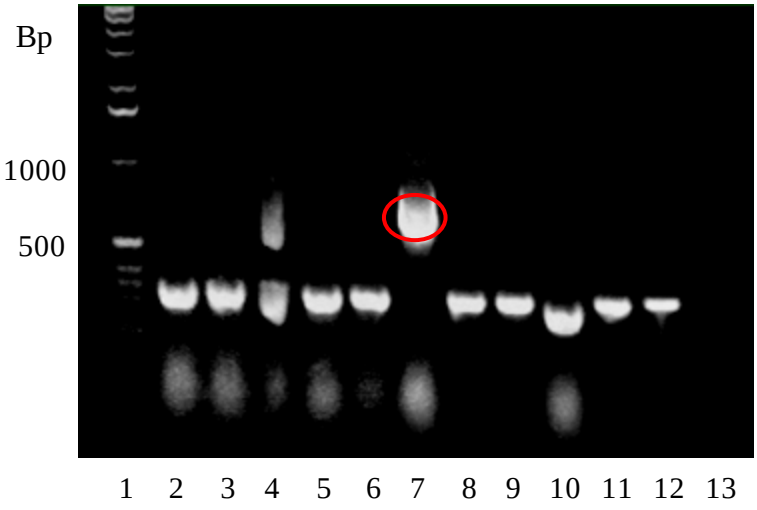


Figure 3.8: Gel image of InFusion cloning of ovine *C4BPB* CDS with pET22b(+) showing one positive colony in lane 7 (circled). Lane 1 = DNA molecular marker X (Roche); Lanes 2-11 = colony PCR (using pET22b forward and reverse primers) of individual colonies from transformed plasmids with lane 7 showing a positive colony including ovine *C4BPB* CDS. All other lanes illustrated negative colonies i.e. pET22b(+) vector contained no *C4BPB* insert. Lane 12 = +ve control (pET 22b vector template); Lane 13 = -ve control (no sequence template).

This plasmid was used to transform BL21 codon plus (DE3) *E. coli* cells (Agilent Technologies) to express the recombinant ovine C4BPB protein. Following induction of recombinant protein production with IPTG, no induced protein bands at the predicted molecular mass of rC4BPB (23kDa) were seen by SDS-PAGE in either the soluble or insoluble phases (Figure 3.9).

As rC4BPB was not successfully expressed using pET22b(+) as a vector system, expression was then attempted using pET SUMO.

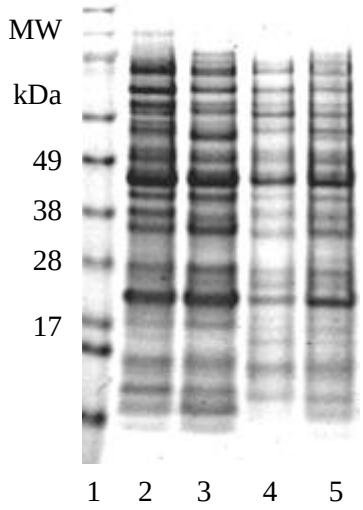


Figure 3.9: Protein expression profiles of ovine rC4BPB in pET 22b separated on a SDS-PAGE gel stained with SimplyBlue Safestain (Invitrogen). Lane 1= SeeBlue marker (Invitrogen); 2= uninduced, soluble fraction; 3=induced, soluble fraction; 4=uninduced, insoluble fraction; 5=induced, insoluble fraction.

3.3.2.2 Using pET SUMO as an expression vector

Ligation of the ovine *C4BPB* sequence into pET SUMO was successful and sequencing of the resulting plasmid confirmed the full sequence was inserted in the correct orientation. This plasmid was used to transform BL21 codon plus (DE3) *E.*

coli cells (Agilent Technologies) to express the recombinant ovine C4BPB protein. Following induction of recombinant protein production with IPTG, the BL21 cells expressed rC4BPB with the pET SUMO fusion protein attached as shown in Figure 3.10. Using pET SUMO has the advantage over other expression systems that subcloning relies on a simple TA cloning step rather than cutting either plasmid or PCR product with restriction enzymes but has the disadvantage that the resulting recombinant protein has a large (14kDa) SUMO fusion protein attached. The fusion protein at ca. 40 kDa therefore represents the rC4BPB, present in the "induced, insoluble" fraction only (Figure 3.10).

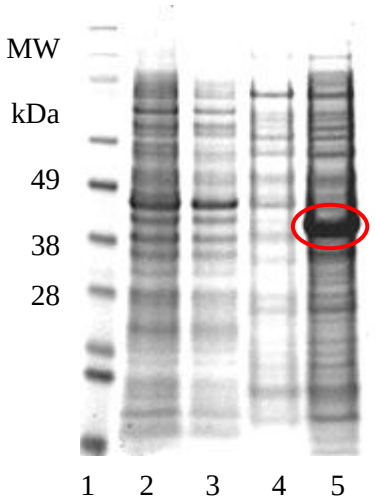


Figure 3.10: Coomassie stained SDS-PAGE gel of rC4BPB expression using pET SUMO expression in BL21 cells, with band of interest circled at ca 40 kDa. Lane 1 = SeeBlue marker (Invitrogen); 2 = uninduced, soluble fraction; 3 = induced, soluble; 4 = uninduced insoluble; 5 = induced insoluble.

3.3.2.2.1 Protein identification by MALDI

The results of the MALDI analysis of the recombinant fusion protein obtained in section 3.3.2.2 confirmed its identity as ovine C4BPB, as shown in Figure 3.11.

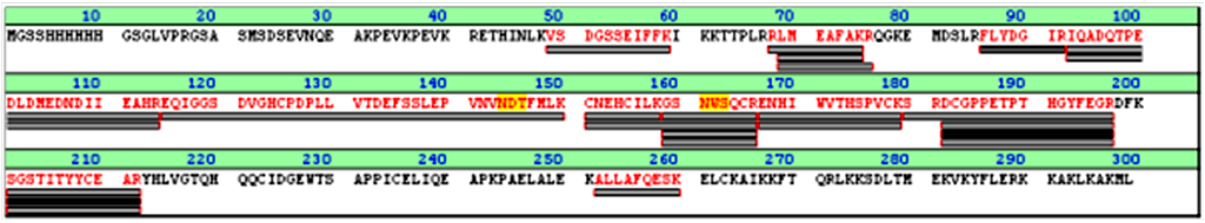


Figure 3.11: Results of MALDI analysis of the excised 40 kDa band from lane 5 in Figure 3.10 confirming the expressed protein identity as rC4BPB. Amino acids in red lettering denote sequence homology between the known ovine rC4BPB sequence and the protein obtained by MS. Shades of grey boxes denote the level of intensity coverage obtained during the MS process from weak (pale grey) to strong (black).

When compared to the predicted ovine C4BPB fusion protein sequence, the intensity coverage was 91.1% and the sequence coverage 51.2%.

3.3.2.2.2 Protein purification and dialysis

The rC4BPB-SUMO fusion protein was purified by nickel affinity chromatography (Figure 3.12) and the band from this protein is evident in the pre-purification sample (lane 2) at approximately 40 kDa and, as a purified recombinant protein, in the three main elution samples (lanes 6-8). Dialysis of the expressed protein after purification was carried out to remove the imidazole from the protein solution and to reduce the urea concentration from 8M (used in the binding buffer for re-suspending the induced pellets for an insoluble protein) to 2M. The protein remained in solution following dialysis and the concentration of the recombinant protein was determined

by BCA assay as 211.4 µg/ml. The total yield therefore was approximately 500µg of rC4BPB from a 50ml culture.

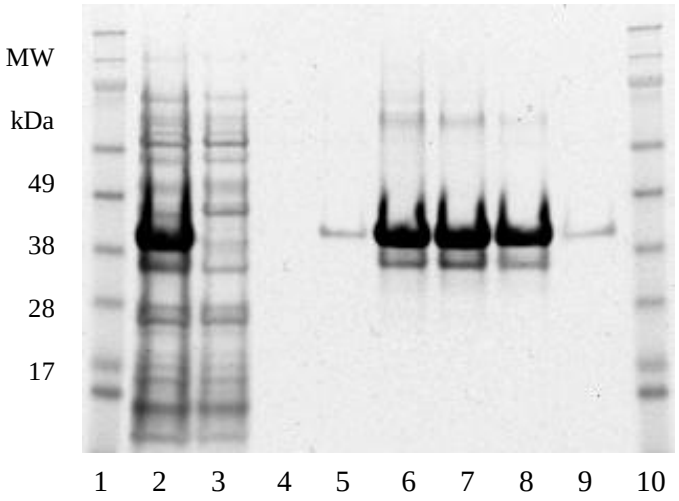


Figure 3.12: Image of SimplyBlue Safestain (Invitrogen) stained SDS-PAGE gel showing eluted fractions during the purification of rC4BPB by nickel affinity column. Lane 1 = SeeBlue marker (Invitrogen); 2 = pre-purification sample; 3 = unbound protein fractions; lanes 4-9 = eluted fractions with lanes 6-8 containing most rC4BPB.

3.3.3 Production of polyclonal antibodies in rabbits against ovine C4BPB

An immunoblot was used (shown in Figure 3.13) to illustrate that each of two rabbits immunised with rC4BPB produced antibodies which bound to the ovine rC4BPB, as bands were obtained at ca 40 kDa in the post-immunisation lanes (2 and 4) which were not present in the pre-immunisation lanes (3 and 5).

When immunoblots of sera from *P. ovis*-infested and non-infested sheep were probed with sera raised in rabbits immunised with rC4BPB, an immunopositive band was obtained at 23 kDa, the estimated MW of native ovine C4BPB which was not present in the negative control as shown in Figure 3.14. A further, unidentified, immunoreactive band was present at ca. 50kDa, which may represent either an oligomer of C4BPB, or a further C4BPB-like protein.

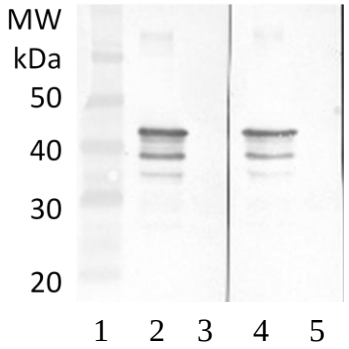


Figure 3.13: Image of immunoblot showing binding of IgG in immunised rabbit sera to ovine rC4BPB. The rC4BPB was separated by electrophoresis on a SDS-PAGE gel, blotted onto nitrocellulose and probed with post 2nd rC4BPB injection sera from each rabbit. Lane 1 = MagicMark (Invitrogen); 2 = rabbit 1 post 2nd injection; 3 = rabbit 1 control sera (prior to immunisation); 4 = rabbit 2 post 2nd injection; 5 = rabbit 2 control sera. Secondary antibody conjugate was swine anti-rabbit HRP IgG (Dako). Visualisation was achieved using ECL Plus (GE Healthcare) and ImageQuant LAS4000 camera (GE Healthcare).

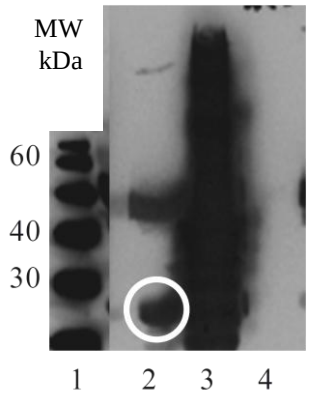


Figure 3.14: Immunoblot of pooled sera from sheep (n = 12) infested with *P. ovis* (6 wk p.i) (TC1), separated by electrophoresis on a SDS-PAGE gel and probed with serum raised in rabbits immunised with rC4BPB (lane 2). Lane 1 = MagicMark (Invitrogen); 3 = over exposed rC4BPB +ve control and lane 4 = -ve (no primary antibody) control for lane 2. The immunoreactive band which putatively represents native ovine C4BPB is circled. The conjugate was swine anti-rabbit IgG HRP (Dako) diluted 1:10000. Visualisation was by ECL (GE Healthcare) and X-ray development.

3.3.4 Development of a C4BPB ELISA

The optimal concentrations of all the individual assay components in the final sandwich ELISA (as a result of the checkerboard ELISAs) are listed in Table A3.2. Three different ELISA formats were compared in the development of an optimised ovine C4BPB ELISA:

1) Direct ELISA

2) Sandwich ELISA with secondary antibody HRP conjugate

3) Sandwich ELISA with biotinylated secondary antibody and streptavidin HRP conjugate

The results of each of these assay formats for the detection of ovine C4BPB in the sera of *P. ovis* infested sheep, at optimal performance, are summarised in Table 3.1.

Table 3.1: Summarised results [± standard error of the mean (SEM)] from the main steps in the ELISA development to detect C4BPB in serum of *P. ovis* infested sheep. "Infested" samples comprised pooled sera from sheep (n = 12) 6 weeks p.i, whereas "control" samples used pooled sera from the same sheep (n = 12) prior to exposure to *P. ovis*. Normalised OD_{450} was calculated by subtracting the negative (no primary antibody) control from the sample OD_{450}.

	Mean normalised OD±SEM Sample from infested sheep	**Mean normalised OD±SEM Control sample**
Direct ELISA	0.232±0.04	0.147±0.07
Sandwich ELISA: HRP conjugated secondary antibody	0.04±0.002	0.02±0.001
Sandwich ELISA: biotinylated secondary antibody	0.570±0.038	0.064±0.077

The differences between the mean normalised OD_{450} using pooled sera from sheep infested with *P. ovis* compared with sera from non-infested sheep were not significant in the direct ELISA and, in the initial sandwich ELISA, the OD_{450} values were very low when an HRP-conjugated secondary antibody was used. When a biotinylated secondary antibody was used with a streptavidin-conjugated HRP at the

established optimal concentration, the OD_{450} increased more than ten-fold and the differences between the infested and control sera were statistically significant, *P*=0.0495, as shown in Figure 3.15. All of the optimisation ELISAs included positive controls using ovine rC4BPB for coating; negative sera controls (sera from rabbits prior to immunisation) and negative (no primary antibody) controls as detailed in section 3.2.4. The mean OD_{450} for the positive controls, used in the ELISAs summarised in Table 3.1, was 2.173±0.262 and that of the negative (no primary antibody) controls was 0.005±0.0001.

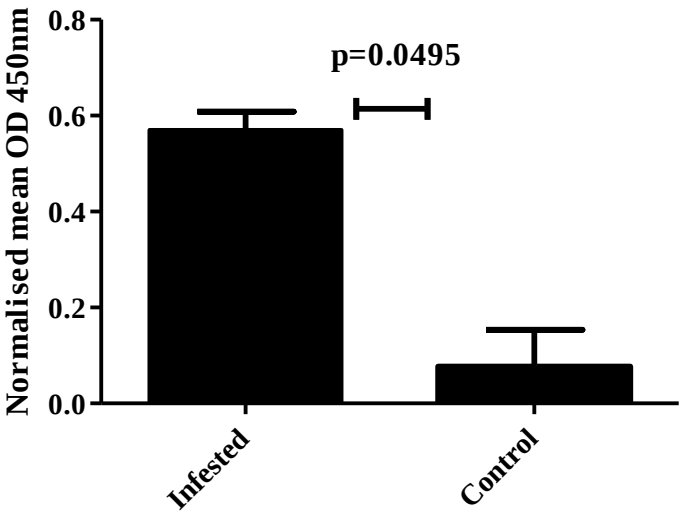


Figure 3.15: Mean normalised OD_{450} obtained from the optimised sandwich ELISA to detect C4BPB in the pooled sera of *P. ovis* infested sheep (n = 12; 6 week p.i) compared with control sheep comprising the same group of sheep prior to exposure to *P. ovis.* Normalised OD was calculated by subtracting the negative control OD (background) from the sample OD. Variance bars represent ±SEM.

3.3.4.1 Use of the sandwich ELISA to quantify ovine C4BPB in sera across a time course of infestation with *P. ovis* and post-treatment in an experimental trial

Pooled sera were used from the TCT1 trial described in A3.1.11 to validate the optimised ELISA. Briefly, blood samples were taken by venous extraction from12 sheep with no previous exposure to *P. ovis* and the sheep were then infested with *P. ovis* mites. Throughout the subsequent 6 weeks of infestation, lesion sizes were measured and blood samples taken by venous extraction weekly. The sheep were treated with the injectable endectocide Dectomax (Pfizer Animal Health) at the end of week 6 post-infestation. Following treatment blood samples were taken by venous extraction twice each week for a month and then weekly for a further 10 weeks.

The results (Figure 3.16) illustrate that the OD_{450} values derived by ELISA were significantly higher when sera from infested sheep were compared to those in pre-infestation samples ($P \leq 0.001$) by 3 weeks post-infestation and OD_{450} values continued to increase until the sheep were treated 6 weeks post-infestation. Within three days of being treated the mean OD_{450} was significantly lower than the mean values before treatment ($P \leq 0.001$) and the mean OD_{450} then continued to decline until it had reached pre-infestation levels by 2 weeks post-treatment. The positive controls mean OD_{450} for the ELISA plates completed in this experiment (coating with ovine rC4BPB and detecting with rabbit anti-serum) was 1.847±0.055.

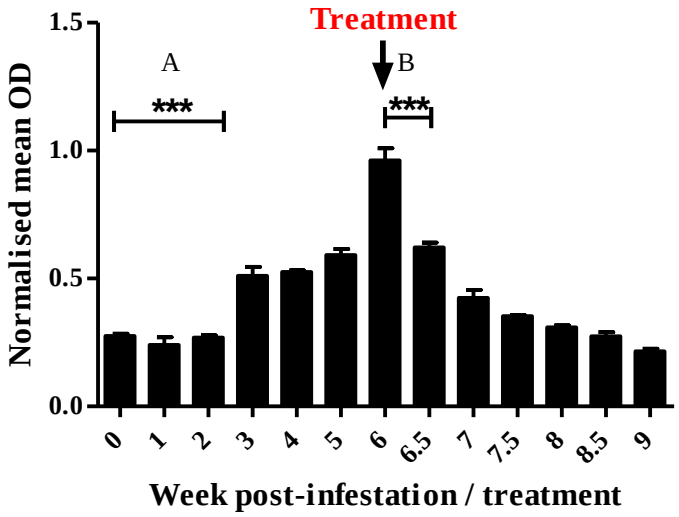


Figure 3.16: Mean normalised OD_{450} obtained from the ovine C4BPB sandwich ELISA using pooled sera sampled from sheep (n = 12) across a time course of infestation with *P. ovis* and post-treatment. Time 0 = pre-infestation. Bar A denotes a significant difference between weeks 0 and 3 and bar B between point of treatment and 3 days post-treatment. ***denotes $P \leq 0.001$ as calculated by Repeated Measures ANOVA (GraphPad Prism). Variance bars represent ±SEM.

3.4 Discussion

An initial PCR amplification using degenerate primers yielded approximately half of the predicted ovine *C4BPB* nucleotide sequence, with the remainder of the sequence being obtained in stages using RACE technology. RACE is a widely used PCR based method employed to capture the 5' and 3' ends of mRNA sequences where only partial sequences have resulted from initial PCR (Schaefer, 1995). It has the advantages of being simple to perform while yielding rapid results, but it often requires extensive optimisation before accurate identification of cDNA ends are achieved (McGrath, 2011). In order to obtain the ovine *C4BPB* sequence, new primers were designed at each step of the RACE sequence extension, based on partial sequences as they were obtained. This increased the specificity and efficiency of each reaction and due to this process of step by step optimisation, the complete sequence was obtained.

The expression of ovine rC4BPB in bacterial cells using pET22b(+) as a vector was unsuccessful. This vector had been selected due to its high success rate in the cloning and expression of recombinant proteins and its ability to maintain the transcription of target genes minimal in the uninduced state (User Protocol TB055, Novagen). Although the *C4BPB* sequence was shown to be inserted in the vector following subcloning, rC4BPB was not expressed on induction. This may have been due to basal expression of T7 RNA polymerase, even in the absence of the IPTG inducer, which induces target gene transcription (User Protocol TB055, Novagen) therefore the rC4BPB expressed in *E. coli* may have been affecting the vector detrimentally. On induction, as the concentration of rC4BPB increased, the protein may have become toxic to some degree to the bacteria, which is a possibility with C4BPB as it

is known to be tightly regulated in humans where it is potentially damaging to cells (Blom et al., 2004).

Ovine rC4BPB was, however, expressed successfully in the pET SUMO vector system, which may be due to the reported effect of the SUMO fusion protein enhancing functional protein production in prokaryotic expression systems and significantly improving protein stability (Panavas et al., 2009). The expressed rC4BPB was insoluble which has been reported previously as being an issue when expressing mammalian proteins in a bacterial system (Demain and Vaishnav, 2009). A further disadvantage of using *E. coli* to express a mammalian protein is that *E. coli* cannot prepare or attach mammalian glycosylation chains (Swartz, 2001). As ovine C4BPB has three glycosylation sites, and it is probable that the rC4BPB expressed in the bacterial system would not have any, rC4BPB is therefore likely to show differences in its tertiary structure when compared with native C4BPB. This differential glycosylation and tertiary structure phenomenon has been cited as the main reason why some antibodies produced from bacterially expressed recombinants fail to bind their target proteins (Demain and Vaishnav, 2009, Vervelde et al., 2002) and also the reason why some recombinant vaccine antigens are ineffective in cases where the native proteins afford protection (Geldhof et al., 2004, 2007, 2008). A similar phenomenon in the present study may account for the sub-optimal binding of antibody raised against rC4BPB to native ovine C4BPB obtained in the Western blot results and low OD_{450} values in the ELISAs in the development stages of the assay. A recent review has also highlighted that it is often difficult to isolate the relative

importance of glycosylation from other post-translational modifications such as protein folding in recombinant antigen immunogenicity (Ellis et al., 2012).

There is clearly a requirement for systems that can modify and glycosylate expressed recombinant antigens in a similar fashion to the native molecule and mammalian expression systems are an attractive alternative to bacterial and yeast-based expression systems for the production of mammalian proteins which require post-translational modifications, such as glycosylation and correct folding (Demain and Vaishnav, 2009). Compared to bacterial expression systems, mammalian systems are expensive, time consuming and difficult to execute (Demain and Vaishnav, 2009) but may have produced better results in this study and should be considered for any future work with C4BPB protein expression.

The results of the direct ELISAs (section 3.3.4 and Table 3.1) showed very low normalized OD_{450} with no statistically significant difference between the OD_{450} of the sera from infested sheep and that of the sera from sheep with no previous exposure to *P. ovis*, suggesting either that the antibody raised in rabbits immunised with ovine rC4BPB had limited specific binding to native C4BPB or that C4BPB was present in very low concentrations in ovine sera.

A sandwich ELISA was therefore designed to increase the specificity and sensitivity of the detection of C4BPB in the sera of sheep infested with *P. ovis*. The technique of using two antibodies raised against the antigen to be detected is a frequently used method of achieving increased sensitivity and specificity (Luo et al., 2012). A commercially available monoclonal C4BPB (mC4BPB) antibody, raised in mice immunised with human rC4BPB, was used as the coating antibody, as this antibody

had previously been shown to cross-react with ovine C4BPB (Chapter 2). A polyclonal C4BPB antibody raised in rabbits against ovine rC4BPB was used as the detection antibody with a swine anti-rabbit conjugated HRP secondary antibody. However, the normalised OD_{450} values for sera from sheep infested with *P. ovis* and control sera from non-infested animals were very low reinforcing the possibility that there were very small quantities of C4BPB circulating in sheep sera. Alternatively there may have been weak binding of the antibody (raised in rabbits against ovine rC4BPB) to native ovine C4BPB. To increase the signal, the secondary antibody HRP conjugate was replaced with a biotinylated secondary antibody coupled with a streptavidin HRP conjugate. This is a commonly used method of increasing the sensitivity of an assay (van Gijlswijk et al., 1996) and in this instance increased the mean OD_{450} of the samples from the sheep infested with *P. ovis* by more than 10-fold. Signal enhancement has recently been used to develop a more reliable diagnostic test for *S. scabiei* in humans. Diagnostic sensitivity has generally been low using current methods of skin scraping due to the low numbers of mites present in ordinary scabies (Walton and Currie, 2007). In a study measuring IgE levels in 140 plasma samples from scabies-infested and control subject groups to a recombinant *S. scabiei* antigen, Sar s 14.3, by dissociation enhanced lanthanide fluorescent immunoassay (DELFIA), high sensitivity and specificity were obtained (Rose and Wall, 2012). The DELFIA assay enhances signal up to 2-fold when compared to ELISA by using a Europium (Eu) labelled antibody instead of an enzyme (PerkinElmer Technical Support). For future C4BPB immuno-assay development, this method may be useful if signal enhancement is required.

The results of the experiment using the improved sandwich ELISA (Figure 3.16) to detect C4BPB in sera of sheep across a time course of infestation with *P. ovis* and after treatment with an injectable endectocide (as described in A3.1.11), suggested that C4BPB was a potential BM for the indication of current disease status in the diagnosis of sheep scab. The mean normalised OD_{450} increased significantly between pre-infestation sera and 3 week post-infestation sera indicating that C4BPB up-regulation in sheep sera occurs in early infestation. However, this was one week later than the specific detection of the host antibody response to the mite antigen Pso o 2 by antibody based ELISA (Nunn et al., 2011) which occurs within 2 weeks of infestation. More importantly for the required function of the BM diagnostic test for sheep scab, the highly significant decrease in mean OD_{450} between the time of treatment and 3 days post-treatment highlighted the potential of C4BPB to provide information on current disease status. The mean OD_{450} at 2 weeks post-treatment was of the same magnitude as that at pre-infestation. This may have a practical implication for assessing disease status on farms where sheep scab has been confirmed. On such farms, the Sheep Scab Order (Scotland) 2010 imposes sheep movement restrictions for 16 days after treatment at which point a veterinary inspection is required to declare the animals/flock free of active infestation. As it is difficult to diagnose a flock as free of active infestation so soon after treatment when relying on clinical observations and skin scraping samples (Bates, 1999c) and the existing assay for diagnosing sheep scab relies on an antibody which can be detected for 3-6 months post-treatment (Bates, 1999c, Ochs et al., 2001, Burgess et al., 2012b), this may be a useful characteristic of the C4BPB response if further validated as a BM for sheep scab.

These initial observations indicate the potential of C4BPB as a BM for sheep scab and also the potential use of the sandwich ELISA as an assay to determine C4BPB levels in sheep sera. However, to progress with this ELISA as a diagnostic tool, further validation and optimisation will be required. In the first instance, many field samples need to be analysed. The gold standard set by the World Organisation for Animal Health (OIC) for the validation of diagnostic assays for infectious diseases is 500 known positive samples, confirmed by skin scraping in the case of sheep scab diagnosis, and 1500 known negative samples as described by the Validation and Certification of Diagnostic Assays determined by the OIC. Further validation of the ELISA would then be required to determine assay sensitivity, specificity, intra- and inter-assay variation and an optimal cut-off point to discriminate between positive and negative ODs (Nunn et al., 2011). An optimal cut-off can be described by a receiver operator characteristic (ROC) curve analysis which optimises the cut-off value required in diagnostic tests based on continuous outputs such as sensitivity and specificity (Pepe, 2003).

It has been widely reported from work in the medical field that method validation is a critical component of BM research and it is often at this point that clinical BM validation fails (Brookes et al., 2010). Stringent assay validation and standardised sampling handling are critical if the variation found inter- and intra-assay is to be minimised and meaningful serum BM concentration changes in healthy and diseased states are to be reported accurately to the clinician (Brookes et al., 2010) and it would therefore be crucial to further validate the diagnostic ELISA on these criteria.

The assay developed in this study relied on one recombinant protein to produce a singleplex sandwich ELISA. However, as C4BPB is an inflammatory protein involved in the regulation of the complement cascade, its increase in concentration in the sera of sheep infested with *P. ovis* is unlikely to be specific to sheep scab. Indeed, the up-regulation of C4BPB has been described in several human inflammatory diseases as previously discussed (Ma et al., 2010, Zadura et al., 2009). It has recently been reported that a "cocktail" of recombinant proteins were useful while developing a diagnostic assay for neosporosis, the major cause of abortion in dairy cattle worldwide (Dong et al., 2012). This study used three recombinant proteins, expressed in *E. coli*, to detect specific antibodies in infected cattle sera. When used in combination in an indirect ELISA, they improved the sensitivity and specificity of the assay compared to when used individually. This is consistent with other studies (Chiswick et al., 2012, Cooper et al., 2007, Komatsu et al., 2011) where a "signature" of BMs or antigens tested for clinical application provided a more accurate platform for diagnostic testing, treatment development and therapy outcomes when compared with a single entity and may be a useful technique to apply in the further development of an ELISA to diagnose current disease status for sheep scab. C4BPB could be used alongside other protein BMs to provide a signature response to sheep scab, or in conjunction with the Pso o 2 assay currently being developed as a diagnostic test for sheep scab (Nunn et al., 2011).

3.5 Conclusion

In this chapter, the ovine *C4BPB* gene was successfully sequenced and a recombinant form of this protein was expressed in *E. coli.* Antibodies, raised in rabbits against ovine rC4BPB, appeared to bind to native C4BPB in Western blots. A direct ELISA using the rabbit antisera against the sera of sheep infested with *P. ovis* did not yield positive results, possibly due to limited binding of the antibody raised against rC4BPB to native C4BPB or to the lack of abundance of C4BPB in sheep sera. The development of a sandwich ELISA, where the use of an additional capture antibody increased the signal sufficiently to amplify the differences in OD_{450} between sera from sheep infested with *P. ovis* and control sera from non-infested sheep, gave promising results. This was further improved by the addition of a biotin/streptavidin detection system which further increased the signal obtained. Evaluation of the ELISA using sera from a time course of infestation with *P. ovis* and post-treatment suggested the potential of C4BPB as a BM for sheep scab as it indicated current disease status, but further evaluation and validation are required before this could be used as a diagnostic tool.

Chapter 4: Evaluation of selected acute phase proteins (APPs) as potential biomarkers (BMs) for sheep scab

4.1 Introduction

4.1.1 Ruminant APPs

Psoroptes ovis infestation in sheep produces an intense cutaneous and systemic inflammatory response involving many aspects of the immune system, including the complement cascade and APR (Van den Broek et al., 2000) as shown by the differential expression of genes involved in the regulation of the complement system, such as *C4BPB* and *C4BPA* in circulating leukocytes, and those involved in the initiation of the APR, such as *IL1β, IL6 and TNF*, in skin biopsies of sheep infested with *P. ovis* (Burgess et al., 2010, Burgess et al., 2012a). The involvement of the complement cascade and APR in the host immune response to *P. ovis* infestation has previously been discussed in Chapter 1. However, as the APR to *P. ovis* infestation in sheep had not previously been characterised, it was decided to investigate the sera concentrations of the two major ruminant APPs; Hp and SAA and also a moderate APP alpha-1 acid glycoprotein (AGP) as potential BMs of sheep scab.

In ruminants, Hp and SAA are considered to be major APPs as their concentrations in sera increase during infection, inflammation or tissue injury by up to 1000-fold for SAA and up to 100-fold for Hp, whereas AGP is considered a moderate APP showing serum increases of up to three-fold during similar inflammatory events (Murata et al., 2004, Petersen et al., 2004). The fold increase from baseline levels is generally higher for SAA than for Hp in mammalian species (Gabay and Kushner, 1999), such as that reported for cattle with bovine respiratory syncytial virus (BRSV)

and mastitis where increased SAA levels were 10-fold higher than that of Hp (Eckersall and Bell, 2010). However, these responses are known to vary between species and disease (Mackiewicz, 1997) and, to date, there has been very little research relating to APPs in sheep (Eckersall and Bell, 2010). It is widely documented however that APPs are highly conserved across species and the amino acid sequence of SAA in sheep shows a high degree of homology with SAA from other mammalian species (Syversen et al., 1994). For example the amino acid sequence identity between bovine and ovine SAA is 70.8% (Uniprot, EMBL-EBI).

The APR in ruminants is unique in that Hp is a major APP which can increase in serum within two days of infection (Eckersall and Bell, 2010) and in sheep, as for cattle, high sera concentrations of Hp and SAA are characteristic of acute inflammatory disease (Horadagoda et al., 1999). In cattle, these APPs are synthesised only in response to inflammation and are present in very low concentrations in the serum of healthy animals (Dobryszycka, 1997). As such, they represent potential BMs and have been used in this context to monitor acute inflammatory disease in cattle, including the diagnosis and prognosis of mastitis, enteritis, pneumonia, peritonitis, endometritis and endocarditis (Murata et al., 2004, Petersen et al., 2004, Bannikov et al., 2011). SAA, in particular, is useful in the diagnosis of mastitis in dairy cattle as a mammary isoform (M-SAA3) of SAA is synthesised in infected mammary glands and can be detected in milk samples enabling subclinical detection of mastitis without the need for invasive sampling (Gerardi et al., 2009).

In ruminants a third APP, AGP has been studied as a potential marker of infection for disease diagnosis. In an experimental model of ovine caseous lymphadenitis (CLA), AGP concentrations in the sera of infected sheep increased to a peak four

times that of the control group, with a more gradual increase and a slower decline than either Hp or SAA suggesting its potential role as a marker of chronic inflammation (Eckersall et al., 2007) .

The three APPs introduced in this chapter have previously been suggested as potential BMs for inflammatory disease in goats (Gonzalez et al., 2008); for diagnosis of infestation with *Sarcoptes scabiei* in Alpine ibex (Rahman et al., 2010) and, recently, the presence of an APR has been established in sheep with clinical scrapie, as determined by increased sera concentrations of Hp, SAA and ceruloplasmin at disease onset suggesting the potential of these APPs as diagnostic BMs (Meling et al., 2012).

4.1.2 The application of APPs as BMs in veterinary diagnosis

Hp and SAA sera concentrations increase rapidly from low circulating levels in response to inflammatory disease, trauma or injury, and as they are present in most body fluids (e.g. serum, urine, saliva, amniotic fluid), they have been used in human medicine as markers for inflammatory disease and in general health profiles for several decades e.g. C-reactive protein (Eckersall and Bell, 2010). The use of an index, involving the combined response of positive and negative APPs with those showing rapid and slow responses, as a prognostic inflammatory and nutritional marker in human cancer has increased the sensitivity and specificity of the test as a clinical marker (Gruys et al., 2006). This index has also been adapted for cattle as an acute phase index (API) and has been used as an indicator of health and welfare at slaughter (Toussaint et al., 1995).

Despite the wide body of research indicating the potential role of these APPs as markers of health and welfare in many domestic animals, they remain under-used in veterinary medicine (Eckersall and Bell, 2010, Cray et al., 2009). This may be due to the difficulties in developing commercially viable antibodies suitable for inclusion in commercial kits, such as producing an antibody against Hp which is a highly glycosylated protein (D. Eckersall, Pers. Comm.); or the assays are too expensive for veterinary practioners to use routinely on commercial farms. Many of the assays that have been used to date are also species-specific, therefore adding to the time and cost constraints of testing (Skinner and Roberts, 1994). Current commercially available assays rely on either ELISA for measuring sera SAA and Hp in cattle (e.g. Alpha Diagnostic International) or a colorimetric assay for Hp analysis based on the binding of Hp to haemoglobin (Hb) (e.g. Tridelta Development Ltd.). This latter assay relies on the innate peroxidase activity of the Hp-Hb complex and the level of peroxidase activity is directly proportional to the quantity of Hp present in the specimen (Eckersall et al., 1999). This indirect method of Hp measurement has the advantage of being relatively quick and easy to analyse and can be performed on all species, but it is expensive and can be inaccurate if the sera samples are haemolysed. However, high throughput Hp analysis is now available due to the development of an automated assay by the commercial laboratory ReactivLab (University of Glasgow) which may increase the commercial use of Hp as a marker of health and welfare in ruminants (D. Eckersall, Pers. Comm.). The ELISAs used for SAA analysis, in comparison, require lengthy processes and are generally species specific; although the SAA ELISA manufactured by Tridelta Development Ltd is predicted to cross

react with SAA in sheep due to the high identity of SAA between these species, according to the manufacturer.

4.1.3 Biological functions of the APPs under evaluation

4.1.3.1 Haptoglobin (Hp)

At the time of this study, although the ovine *Hp* gene had not been sequenced, the bovine sequence was known. Bovine Hp is 401 amino acids in length and has a predicted MW of ~45 kDa (Uniprot, EMBL-EBI). It is a highly glycosylated serum glycoprotein complex consisting of a tetramer of two alpha and two beta chains and, although expressed predominantly by hepatocyte cells, it can also be expressed by adipocytes, lung cells and in mammary tissue (Dobryszycka, 1997, Hiss et al., 2004). A major function of Hp is the binding of free haemoglobin to reduce oxidative damage associated with haemolysis (Yang et al., 2003) and the subsequent loss of iron through the kidneys. Haptoglobin also has immune response regulatory functions through its inhibitory effect on granulocyte chemotaxis thus allowing granulocyte aggregation, promotion of phagocytosis and bactericidal activity by association with iron-binding microbial proteins thereby providing a mechanism to assist the destruction of intracellular pathogens (Dobryszycka, 1997).

4.1.3.2 Serum amyloid A (SAA)

Unlike *Hp*, the ovine *SAA* gene had been sequenced at time of study and the protein comprises 112 amino acids with a predicted MW of ~ 13 kDa (Uniprot, EMBL-EBI). SAA is an APP, expressed in the liver, and two isoforms are produced in sheep (Syversen et al., 1994). Its functions, although not fully understood, are related to the APR and host defence during inflammatory conditions (Murata et al., 2004). Recent studies confirmed that mRNA encoding SAA was predominantly expressed in the

liver, but other organs, such as lung, mammary gland, uterus, and the gastro-intestinal tract, showed moderate SAA mRNA expression, supporting the hypothesis that SAA has a possible role in innate defence against invading pathogens (Berg et al., 2011). There are two reported main functions of SAA in vertebrates: induction of collagenase and matrix metalloproteinases 2 and 3 which are important enzymes for repair processes following tissue damage; and the ability to act as a chemoattractant for immune cells such as monocytes, leukocytes, mast cells and T lymphocytes (Uhlar and Whitehead, 1999).

4.1.3.3 Alpha-1 acid glycoprotein (AGP)

The ovine *AGP* gene had not been sequenced at the time of study, but the bovine AGP protein has 202 amino acids (Uniprot, EMBL-EBI) and has a predicted MW of ~ 23.5 kDa. It is also expressed by hepatocytes and secreted into the blood where it has two major functions: firstly it is an effective binding protein and involved in the transport of molecules such as heparin, histamine, serotonin and steroids (Fournier et al., 2000); and secondly it is an immune regulatory protein, contributing to the maintenance of homeostasis by reducing tissue damage associated with inflammation (Murata et al., 2004). As with other ruminant APPs, veterinary diagnostic use has been limited due to the lack of commercially available assays. However, as new techniques are developed this may change as, for example, a specific ovine AGP radial immunodiffusion assay is available for commercial use (Tridelta Development Ltd).

The aims of this chapter were:

- To investigate serum concentrations of Hp and SAA, two of the major ruminant APPs, in response to *P. ovis* infestation in sheep. This was performed using commercially available assays to assess their potential in the diagnosis of current disease status in sheep scab outbreaks and in particular, to assess their levels following treatment for sheep scab.
- To indicate the specificity of Hp and SAA to *P. ovis* infestation by measuring their serum concentrations during other common acquired diseases and conditions of sheep in the UK.
- To evaluate AGP as a potential BM for sheep scab.
- To analyse APP profiles following acaricidal treatment of sheep for *P. ovis* infestation and to compare the duration of these responses to that of the ovine IgG response to the *P. ovis* antigen Pso o 2.

4.2 Materials and Methods

4.2.1 Samples used in APP analysis

4.2.1.1 Sera samples

The serum levels of Hp and SAA during infestation of sheep with *P. ovis* were measured using samples from the experimental trials described in Chapter 2, section 2.2.3.6 i.e. TC1, TC2 and TC3, and in Chapter 3, A3.1.11 (TCT1). Briefly, these sera were derived from sheep which had samples taken by venous extraction prior to infestation, then weekly post-infestation for 6 weeks (TC1, TC2 and TC3) and additionally after treatment with an injectable endectocide (Dectomax, Pfizer Animal Health) for TCT1. The primary infestation and subsequent treatment performed in trial TCT1 was followed by a secondary infestation and treatment component, as described in Chapter 3, A3.1.11.

In addition, the samples listed below were included to test the APP response during other common diseases and conditions of sheep:

- sera from individual sheep (n = 6) experimentally infected with multiple species of endemic gastro-intestinal nematodes (GINs), including *T. circumcincta* and *Nematodirus battus*, from the MRI Firth Mains Farm;
- sera from an experimental infestation of chewing (*Bovicola ovis*) (n = 4) and sucking (*Linognathus spp*) (n = 4) lice, kindly supplied by Prof. Neil Sargison, The Royal (Dick) School of Veterinary Studies, University of Edinburgh;
- sera from sheep (n = 6) during a natural infection with the liver fluke *Fasciola hepatica* from Minsca Farm, Dumfriesshire and kindly supplied by Dr H. MacDougall, MRI;

- sera from sheep (n = 10) during early gestation then from late gestation in the same sheep from MRI and kindly supplied by Prof. G. Entrican;
- sera from sheep infected experimentally with orf virus (n = 6) (kindly supplied by Dr C. McInnes) and Johnes disease (n = 6) kindly supplied by Dr C. Watkins from MRI;
- sera from sheep clinically confirmed by examination with field-acquired sheep scab infestations (n = 12). Sera were available for these animals pre-infestation, at point of clinical diagnosis and two months post-treatment from a natural outbreak of sheep scab at the MRI Firth Mains Farm (Burgess et al., 2012b).

4.2.1.2 Lesion size measurements

Lesion size measurements were recorded weekly for the TC1 trial and are shown in Chapter 2, Table 2.1. They were also recorded weekly for the TCT1 trial detailed in A3.1.11 by measuring the length and width of the main lesion on each sheep and recording the result as average mean lesion area (cm^2) ± SEM as shown in Figure 4.1.

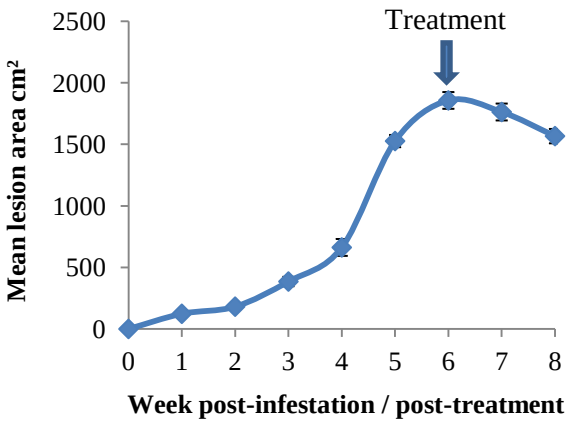


Figure 4.1: Weekly mean lesion areas (±SE) for sheep (n = 12) from the TCT1 trial (A3.1.11) over a 6 week time course of infestation with *P. ovis* and 2 weeks post-treatment with an injectable endectocide. *All animals were treated with Dectomax (Pfizer Animal Health) at 6 weeks p.i.

4.2.2 Haptoglobin (Hp)

SDS-PAGE, MALDI analysis and Western blot analysis were performed, as detailed below, to assess the qualitative relationship between serum Hp concentration and sheep scab lesion size development through a time course of *P. ovis* infestation. Following this a quantitative Hp assay was used to further define the relationship between serum Hp concentrations and current disease status in sheep.

4.2.2.1 Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE electrophoresis was used to separate pooled sheep sera (TC1 experimental *P. ovis* infestation study as described in Chapter 2, section 2.2.3.6). Sheep sera (diluted 1:10) were added to 4X NuPage LDS sample buffer (2.5µl) (Invitrogen) and NuPage sample reducing agent (1µl) (Invitrogen) to a total volume of 10µl and incubated at 70°C for 10 mins. Electrophoresis was performed, using

NuPage BisTris 4-12% Polyacrylamide gels (Invitrogen) with MES buffer as described in A2.1.1 at 250V for 35 mins in a PowerEase 500 electrophoresis cell (Invitrogen). The molecular weight marker used was SeeBlue Prestained Standard (Invitrogen).

4.2.2.2 MALDI analysis

Gels were stained in SimplyBlue Safestain (Invitrogen) following the manufacturer's protocol. The bands of interest, identified as they increased in intensity through the time course of infestation, were excised from the lane containing week 5 post-infestation sera and mass spectrometry (MS) using MALDI performed at MRI Proteomics facility as described in Chapter 3, section 3.2.2.2.1.

4.2.2.3 Hp Western blot analysis

SDS-PAGE and immunoblotting were performed, as described in Chapter 2, section 2.2.3, using samples from two individual sheep in the TC1 trial described in section 2.2.3.6. Sheep 1 had a naturally resolving lesion, which was at maximum size at week 4 post-infestation, whilst the lesion from Sheep 5 showed a gradual expansion over the time course of infestation (Chapter 2, Table 2.1). The primary antibody used for detecting Hp in the electrophoresed serum samples was a rabbit polyclonal anti-human Hp (Abcam) and was used at a dilution of 1:1000. The secondary antibody conjugate used was a swine anti-rabbit IgG HRP (Dako) and was used at a dilution of 1:2000.Visualisation was by ECL Plus (GE Healthcare) using the ImageQuant system (GE Healthcare) as described in Chapter 2, section 2.2.3.5.

4.2.2.4 Hp assay

Haptoglobin concentration was quantified in the sera of sheep from the experimental trials TC1 (n = 6) and TC2 (n = 8) described in Chapter 2, section 2.2.3.6, and TCT1 (n = 12) described in A3.1.11. Haptoglobin concentration was also quantified in the samples described in section 4.2.1.1 from sheep with other common diseases and conditions. All sera samples were analysed by ReactivLab (Glasgow University) using a colorimetric assay measuring absorbance at 600nm. The analyser was an ABX Pentra 400 (Horiba Medical) with a limit of detection for Hp of 0.02 mg/ml. A high Hp standard of 1.48 mg/ml was used therefore samples with Hp values above this value were automatically diluted. All samples were tested in duplicate.

4.2.3 Serum Amyloid A (SAA)

As for Hp, a qualitative assessment was made of the relationship between SAA band intensity and lesion size development by Western blot analysis using a commercially available antibody raised in rabbits against human SAA. Following this, a quantitative analysis was performed by SAA ELISA (Tridelta Development Ltd.)

Western blot analysis was performed as described in section 4.2.2.1. The sera samples used were from the time course trial (TC1) as described in section 2.2.3.6, from two individual sheep (animals 1 and 6) as described in section 4.2.2.3 except sera from sheep 6 was used instead of sheep 5, as it also had an expanding lesion throughout the period of infestation. The primary antibody was a rabbit polyclonal anti-human recombinant SAA (rSAA) (Abcam) and was used at a dilution of 1:500 as established by titration. The conjugate used was a swine anti-rabbit HRP IgG (Dako) and was used at a dilution of 1:2000. X-ray films were used for image

development, which were scanned and band densities estimated using the Quantity One software 4.6.2 (Bio-Rad) as described in Chapter 2, section 2.2.3.5.

A commercially-available sandwich ELISA kit (TP805B, Tridelta Development Ltd) was then used to quantify SAA in sheep sera. This assay was based on a monoclonal anti-bovine SAA antibody raised in mice which had been previously shown by the manufacturers to cross-react with ovine SAA (Tridelta Development Ltd). The manufacturer's protocol was followed using the same sera as for Hp described in section 4.2.1.1, with the addition of TC3 sera. Briefly, 96-well plates were pre-coated with an anti-bovine SAA monoclonal antibody raised in mice and samples, including standards of known SAA concentration included with the assay, were added to the wells along with a biotinylated anti-bovine SAA antibody, before adding a streptavidin-HRP conjugate. A 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate was added and the reaction stopped using the stop solution provided with the kit and the OD measured at 450nm (OD_{450}) using a 630nm filter as reference, on a Sunrise™ 96-well plate absorbance reader (Tecan). Samples were tested from individual animals in duplicate, including the standard samples included with the ELISA kit and all serum was diluted 1:500 in sample diluent buffer (Tridelta Development Ltd). The upper level of the range of the bovine standards used was 300 ng/ml therefore all sera samples showing SAA values above this level, were further diluted as required and re-analysed.

4.2.4 Alpha-1 acid glycoprotein (AGP)

In the absence of a suitable quantitative commercially-available assay at the time of study, AGP was evaluated using Western blot analysis, which was performed as

described in section 4.2.2.1 and Chapter 2, section 2.2.3. The primary antibody was a mouse monoclonal anti-bovine AGP (Abcam) and was used at a dilution of 1:500. The secondary antibody conjugate was a rabbit anti-mouse IgG HRP (Dako) used at a dilution of 1:2000. Visualisation was by ECL Plus (GE Healthcare) and X-ray film.

4.2.5 Analysis of antibody levels to the specific *P. ovis* antigen Pso o 2

To allow comparison of the ovine IgG response to Pso o 2 with serum levels of Hp and SAA post-infestation with *P. ovis* and post-treatment with an injectable endectocide, an optimised ELISA, currently being commercialised for the diagnosis of sheep scab (Nunn et al., 2011) and described in the general introduction of this thesis, was used. Serum samples from the TCT1 experimental trial (described in A3.1.11) were assayed and due to previously reported persistence in antibody levels after treatment (Ochs et al., 2001, Burgess et al., 2012b) serum samples were analysed to 14 weeks post treatment after primary infestation. The ELISA was performed as previously described (Nunn et al., 2011) with the following modifications: All sera samples were assayed for individual animals in duplicate and were diluted 1:200 in PBS with 0.5% Tween80 prior to use. Positive, inter-plate controls were hyper-immune serum samples used in the validation of the ELISA obtained from sheep (n = 2) which had been re-infested with *P. ovis* for 6 weeks following a primary infestation (6 weeks) and effective treatment. Negative controls consisted of the pre-infestation sera from the TCT1 trial sheep.

4.2.6 Statistical analysis

Initial statistical analyses of results of the time course trials, TC1, TC2, TC3 and TCT1 for Hp and SAA were performed using repeated measures one-way ANOVA

with Tukey's post-hoc test and performed in Graph Pad Prism (Version 5.05, GraphPad Software Inc).

Further statistical analysis was performed by Biomathematics and Statistics Scotland (BioSS) using the R Statistical Package (R Core Team, 2012) and the Hp, SAA and Pso o 2 assay results from the TCT1 primary infestation and treatment trial (described in A3.1.11). The statistical analysis examined the relationship between Hp, SAA and Pso o 2 relative to each other and to disease progression with time and lesion size development. Briefly, this assessment included the following factors:

- analyzing the correlation between the duplicated results from each of these assays;
- identifying best fit models to describe Hp, SAA and Pso o 2 trends over the time course of infestation and post-treatment. The best fit model for describing the response of anti-Pso o 2 to *P. ovis* infestation and treatment was that log mean OD_{450} increased linearly over time. This model showed poor fit to the Hp and SAA results so in these analyses the animals were described as being "normal" as in day 0 (pre-infestation) or "elevated". As the results showed a normal distribution, all values above the 0.999 quantile of the distribution were considered to be elevated, giving a 99.9% specificity and undefined sensitivity;
- calculating half life values (time taken for the assayed value to decrease by half following treatment). A generalised linear mixed model was used to describe the exponential decay of the BMs and Pso o 2 and incorporated a random effect of animal on the decay constant, and the initial value. Thus the

decay constant (and the half-life derived from it) represented the mean of all the population of animals from which the group sampled came. By using all the data, a more robust estimate of the half-life was obtained;

- calculating optimised cut-off values, where the objective was to obtain as high sensitivity and specificity as possible to keep false results to a minimum. This was achieved using a generalised linear mixed model for binomial data, with a logit link which required the use of the library "lme4" (Bates et al., 2011). This model used day 0 results as known negatives and week 6 post-infestation as known positives;
- calculating the numbers of sheep which would require to be tested at selected levels of disease prevalence using the obtained cut-off values. Weeks 0, 1, 2, 3 post-infestation and days 10 and 14 post-treatment were taken as Hp having no *P. ovis* induced response and weeks 5 and 6 post-infestation, plus days 3 and 7 post-treatment when there was a response. SAA was calculated in the same way, except there were no post-treatment days where it was considered there was a *P. ovis* induced SAA response. The individual BM test sensitivity and specificity obtained were applied to selected levels disease prevalence in flocks of 20, 50, 100 and 200 sheep and the numbers of sheep were estimated which would require testing using this model.

4.3 Results

4.3.1 Haptoglobin

4.3.1.1 SDS-PAGE

The results of the SDS-PAGE analysis of the TC1 sera across a time course of infestation are shown in Figure 4.2. This image identified that the band densities of two proteins at MWs of 17 and 38 kDa increased through the time course of infestation with *P. ovis*, in particular in lanes 7 and 8, depicting sera from week 5 and 6 post-infestation. To establish the identity of these two proteins, bands at 17 and 38 kDa from week 5 post-infestation were excised from the gel for MALDI analysis.

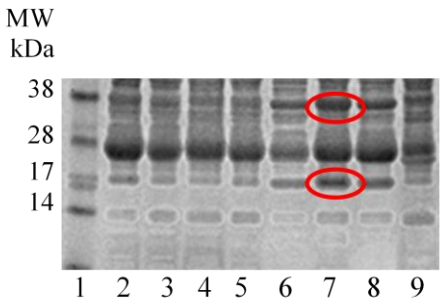
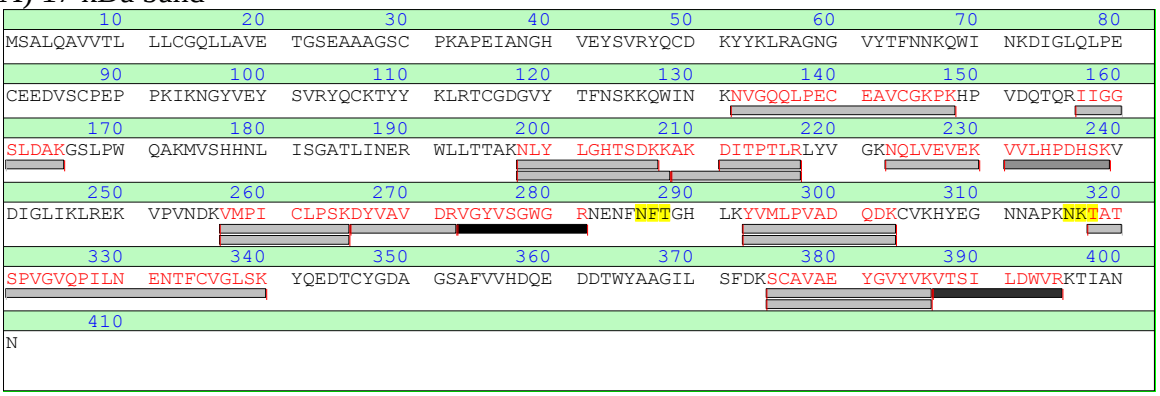


Figure 4.2: Image of SDS-PAGE gel of pooled sera from sheep (n = 6) infested with *P. ovis* over a 6 week time course (TC1) and stained with SimplyBlue Safestain (Invitrogen). Bands excised for MALDI analysis at 17 and 38 kDa are circled in red. Lane 1 = SeeBlue MW Marker (Invitrogen); Lane 2 = pre-infestation sera; Lane 3 = week 1 p.i sera; Lane 4 = week 2 p.i sera; Lane 5 = week 3 p.i sera; Lane 6 = week 4 p.i sera; Lane 7 = week 5 p.i sera; Lane 8 = week 6 p.i sera; Lane 9 = hyper immune sera.

4.3.1.2 MALDI

MALDI analysis was performed by MRI Proteomics on the bands excised from the SDS-PAGE gel (Figure 4.2, lane 7 at 17 and 38 kDa) and the results are shown in Figure 4.3, which identified both samples as fragments of Hp. As the ovine *Hp* gene had not previously been sequenced, the closest match to sample A (17kDa band), when a Mascot (Matrix Science) database was interrogated was *Capra ibex* Hp with sequence coverage of 35.7% and intensity coverage of 79.7%. For sample B (38 kDa) the closest match from the Mascot database was *Cervus elaphus* Hp with an intensity coverage of 11.3% and sequence coverage of 14.5%. Sample A was given a Mascot score (Probability Based Mowse Score) of 127 and sample B a score of 202, where protein scores of >70 are considered to be significant ($p<0.05$).

A) 17 kDa band



B) 38 kDa band

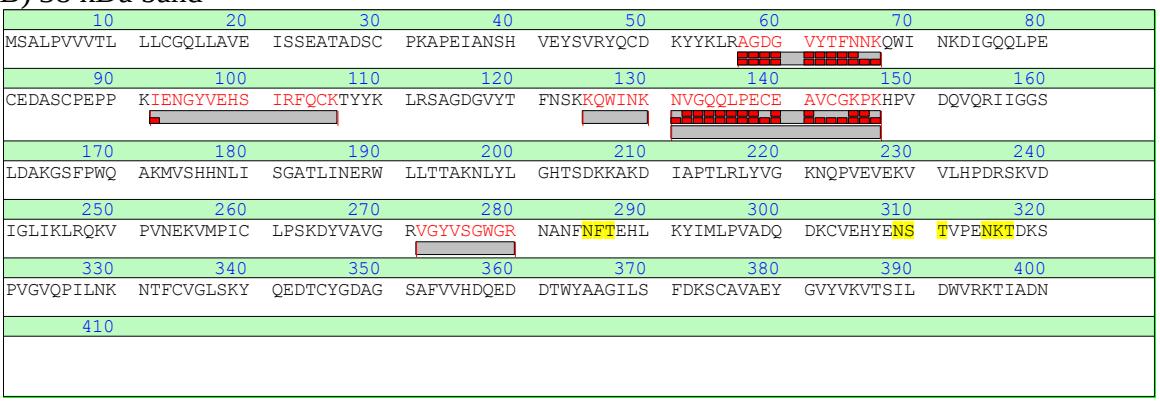


Figure 4.3: MALDI analysis results from the excised 17 kDa and 38 kDa bands (lane 7 in Figure 4.2) confirming the band identities as Hp fragments. Amino acids in red lettering denote sequence homology between the Hp sequence of the closest matched species and the protein fragments obtained by MS from the excised gel bands. Shades of grey boxes denote the level of intensity coverage obtained during the MS process from weak (pale grey) to strong (black).

4.3.1.3 Western blot analysis

Western blot and densitometry analysis was performed to compare the band densities of Hp with lesion size development in two sheep as described in section 4.2.2.3 through a time course of infestation. The results indicated that as the lesion size increased the band density of Hp increased and as the lesion resolved the intensity of the bands decreased (Figure 4.4).

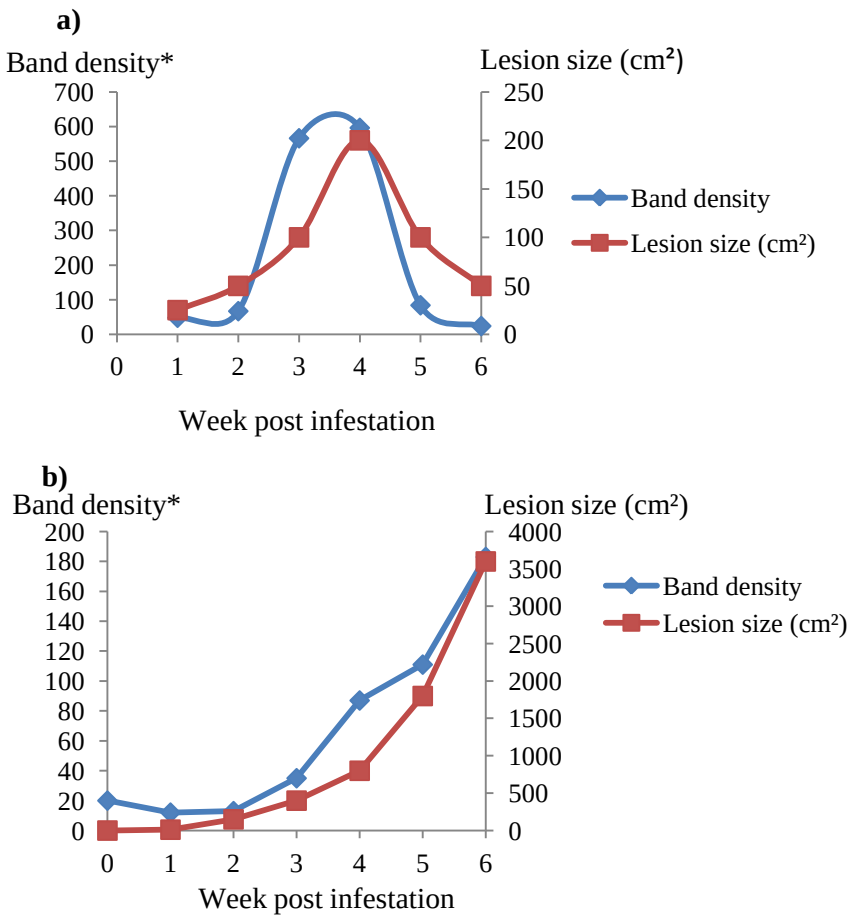


Figure 4.4: Densitometry analysis (ImageQuant) from Western blot images using: **a)** the serum (TC1) from a sheep infested with *P. ovis* where the lesion resolved naturally after 4 weeks (Sheep 1) and **b)** serum from an infested sheep (TC1) where the lesion had continued to expand during the 6 week time course (Sheep 5). Immunoblots were probed with a rabbit polyclonal anti-human Hp antibody (Abcam) and the secondary antibody conjugate used was a swine anti-rabbit IgG HRP (Dako). The image was visualised by ECL Plus and ImageQuant and band densities (at 45 kDa) were quantified by ImageQuant analysis. *Band density was estimated by ImageQuant TL software.

4.3.1.5 Haptoglobin Assay

A colorimetric Hp assay (ReactivLab) was used to analyse sera Hp from two experimental *P. ovis* infestation trials (TC1 and TC2 as described in Chapter 2, section 2.2.3.6) and the results are shown in Figure 4.5 (data tabulated in Table A4.1).

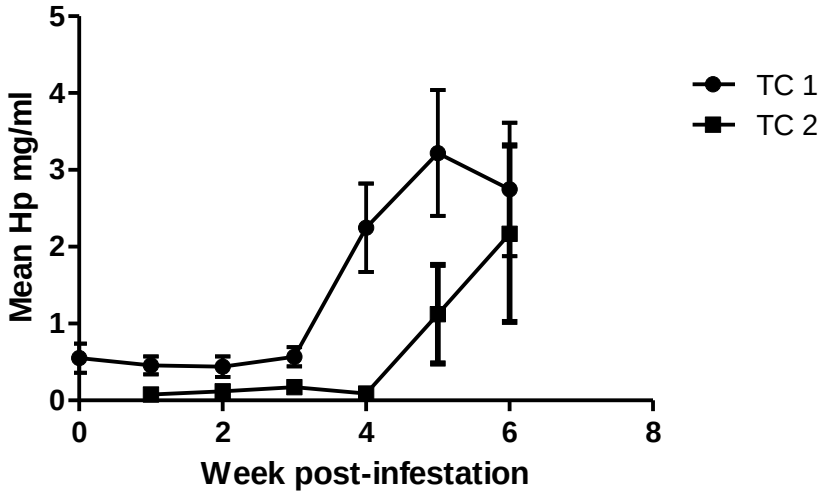


Figure 4.5: Mean sera Hp concentrations (±SE) (n = 6 TC1; n = 8 TC2) as determined by a colorimetric Hp assay (ReactivLab) across a 6 week time course of *P. ovis* infestation in two experimental trials (TC1 and TC2). Samples from individual animals were analysed in duplicate. No sample was available for time 0 in the TC2 trial.

Statistically significant increases in mean Hp concentration in TC1 were observed between weeks 0 and 5; 1 and 5; 2 and 5; and 3 and 5 ($p \leq 0.01$). However, there were no statistically significant differences between any of the time points in TC2 due to the large between animal variations in sera Hp, particularly at higher Hp concentrations. The results of the experimental trial TCT1 (12 sheep infested over a

6 week time course, treated and left for 14 weeks prior to another 6 week secondary infestation) are shown in Figure 4.6 and tabulated in Table A4.2.

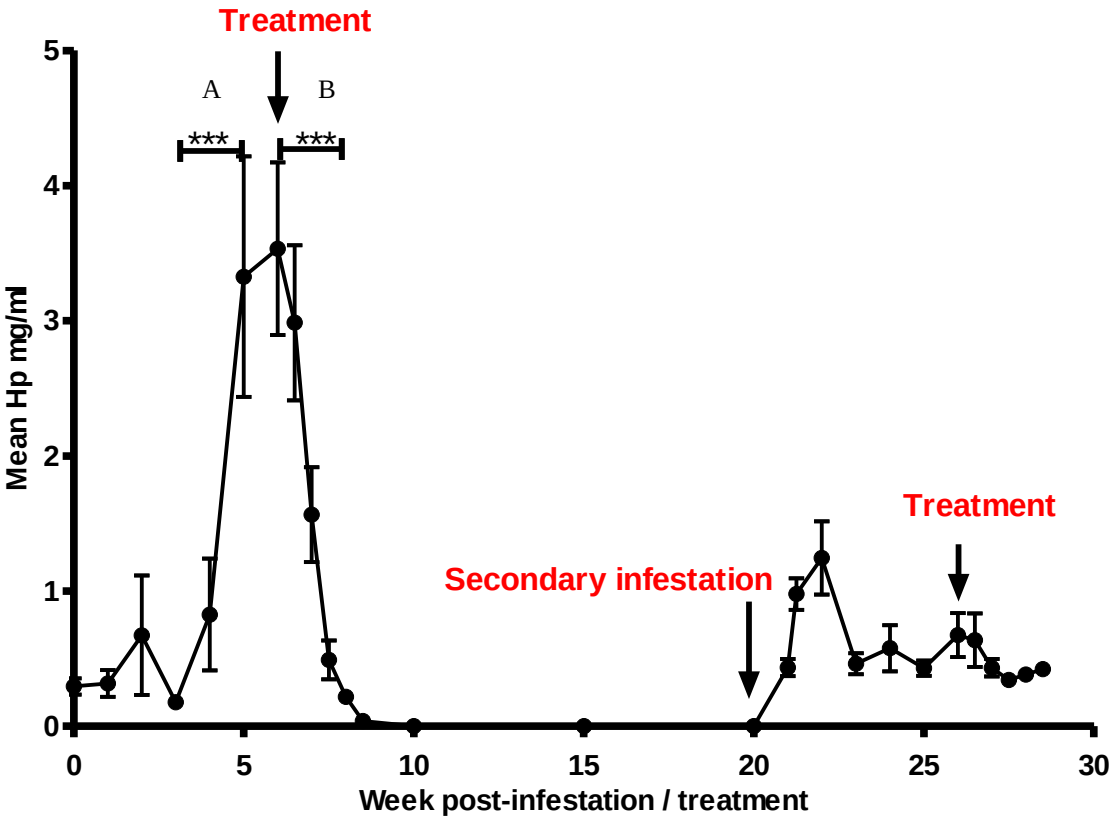


Figure 4.6: Mean Hp concentrations (±SE) through the time course of primary infestation with *P. ovis* and post-treatment over 14 weeks, followed by secondary infestation for a further 6 weeks and post-treatment for 2.5 weeks. Sera samples (n = 12) from individual sheep in the experimental trial TCT1 (described in A3.1.11) were analysed in duplicate using a colorimetric Hp assay (ReactivLab). Bar A denotes a significant difference between weeks 4 and 5 post-infestation and B between point of treatment and 1 week post-treatment. *** $p \leq 0.001$

The mean Hp concentration pre-infestation was 0.295±0.060 mg/ml and Hp levels did not increase significantly during the primary infestation until after 4 weeks p.i. Between weeks 4 and 5 p.i, Hp levels increased from 0.828±0.414 mg/ml to

3.327±0.890 mg/ml ($p \leq 0.001$). Following treatment of the primary infestation, mean Hp concentrations fell significantly ($p \leq 0.001$) from 3.533±0.638 mg/ml at the point of treatment to 1.566±0.351 mg/ml one week post-treatment and had returned to pre-infestation levels between 10 and 14 days after treatment.

The Hp profile, illustrated in Figure 4.6, for the secondary infestation showed that serum Hp levels increased earlier to a challenge infestation with *P. ovis.* The maximum Hp concentration measured during the secondary infestation was at 2 weeks post-infestation (week 22) with a value of 1.246±0.270 mg/ml. Hp levels had doubled by 24 hours p.i in the secondary infestation compared with the much slower increase in levels during the primary infestation. By week 3, following secondary infestation, Hp levels had reduced to pre-infestation levels and they remained at low levels for the remainder of the trial.

The results of the statistical analysis using the R statistical package (R Core Team, 2012) (described in section 4.2.6) to define the nature of elevation and half-life of Hp concentrations in sera are shown in Table 4.1. The proportion of animals showing elevated Hp started to increase between weeks 4 and 5 post-infestation and all except one had returned to normal by 10 days post-treatment. The half life of Hp post-treatment was calculated as 2.3 days [95% confidence interval (CI) = 1.9-2.9]. The optimised range below which an animal was considered "normal" or above it "elevated" was 0.445-1.260 mg/ml for Hp and as all the values within that range gave the same outcome for specificity and sensitivity, a cut-off of 1.26 mg/ml was selected for this analysis, giving a sensitivity of 0.83 and a specificity of 1.0.

Table 4.1: Distribution of animals (n = 12) after statistical analysis using the R statistical package (R Core Team, 2012) into normal and elevated groups for Hp sera concentration across the time course of primary infestation and treatment (TCT1 trial). *All sheep were treated with an injectable endectocide at week 6 p.i.

Week	0	1	2	3	4	5	6*	6.5	7	7.5	8
Normal	12	11	11	11	10	4	2	2	7	11	12
Elevated	0	1	1	1	2	8	10	10	5	1	0

Based on this cut-off value denoting the serum Hp level of a "normal" or an "elevated" animal, the number of sheep which it would be considered necessary to test in a flock is shown in Table 4.2. These results indicate that at higher disease prevalence fewer animals in a flock would require testing and the proportion of animals in a flock which would require testing decreased as the flock size increased up to 200. In addition, irrespective of the flock size analysed, the probability of obtaining false positive results in sheep scab negative flocks decreased as disease prevalence increased.

To further establish Hp concentration in the sera of sheep known to be sheep scab negative, 19 sera samples from sheep scab naive animals were tested for Hp by colorimetric assay (ReactivLab) and the mean Hp concentration was determined as 0.61±0.186 mg/ml. This was a higher mean than that established in the pre-infestation results described in Figure 4.6 and Table A4.2 which showed a mean Hp

concentration of 0.295±0.060 (n = 12), indicating a range of Hp serum levels in animals prior to infestation with *P. ovis*.

Table 4.2: Statistical analysis of Hp results to indicate the number of sheep in a flock which would require testing at selected levels of disease prevalence. This analysis was carried out using R statistical package (R Core Team, 2012) using previously defined test values of sensitivity (0.83) and specificity (1). Sera Hp results from the TCT1 primary infestation and treatment trial were used in this analysis. *Level of accuracy refers to the true detection rate in a positive flock. ** Probability that a negative flock will test positive. NA = not applicable.

FLOCK SIZE	DISEASE PREVALENCE (%)	*TEST ACCURACY	NO. ANIMALS REQUIRED TO TEST	**PROBABILITY OF DETECTION IN NEGATIVE FLOCKS
20	1	0.95	**20**	NA
	5		**20**	NA
	10		**20**	NA
	20		**16**	0.55
50	1	0.95	**42**	0.88
	5		**34**	0.82
	10		**26**	0.73
	20		**16**	0.55
100	1	0.95	**49**	0.91
	5		**36**	0.83
	10		**26**	0.73
	20		**16**	0.55
200	1	0.95	**53**	0.93
	5		**37**	0.84
	10		**26**	0.73
	20		**16**	0.55

4.3.2 Serum Amyloid A (SAA)

4.3.2.1 SAA Western Blotting

To clarify the relationship between serum SAA levels and sheep scab infestation, Western blot analysis was performed using individual sera samples from the time course trial (TC1) where lesion sizes were recorded on a weekly basis (Chapter 2, Table 2.1). The densities of bands obtained in the Western blots, at the estimated MW of SAA (14 kDa), were measured at each time point during infestation using Quantity One software (Bio-Rad). This enabled comparisons to be made for each sheep as lesions grew or resolved as shown in Figure 4.7 which identifies a clear relationship between SAA band density and lesion size i.e. band density increased as lesion size increased and decreased as lesions resolved.

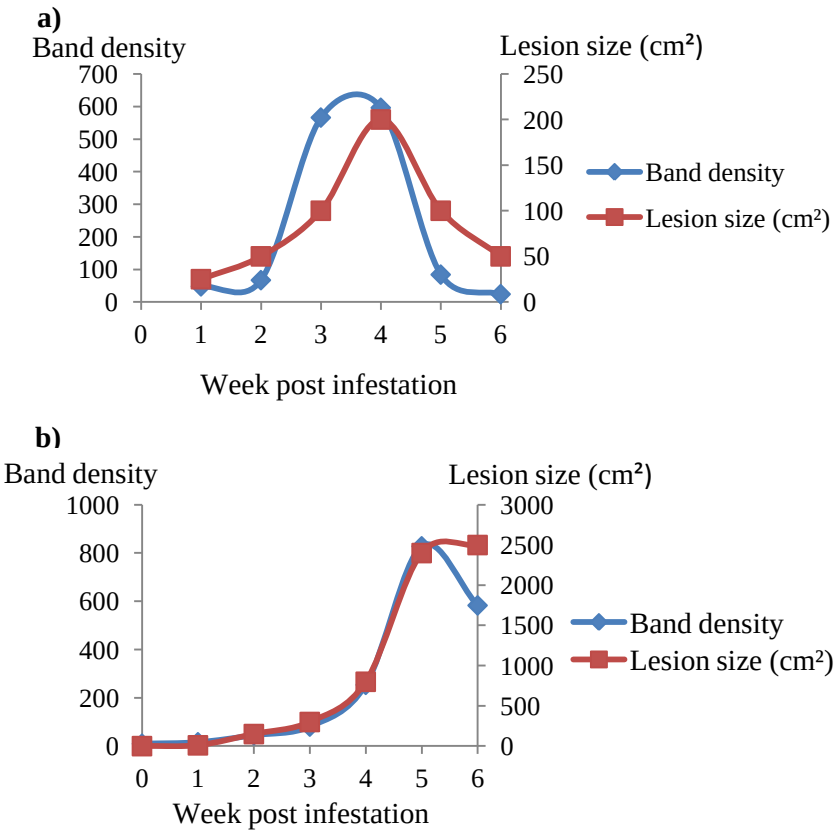


Figure 4.7: Densitometry analysis (Quantity One software, Bio-Rad) from Western blot images using: **a)** the serum (TC1) from a sheep infested with *P. ovis* where the lesion resolved naturally after 4 weeks (Sheep 1) and **b)** serum from an infested sheep (TC1) where the lesion had continued to expand during the 6 week time course (Sheep 6). The blots were probed with an anti-SAA antibody raised in rabbits against human rSAA (Abcam) and the secondary antibody conjugate was swine anti-rabbit HRP IgG (Dako). The blot was visualised using ECL Plus (GE Healthcare).

4.3.2.2 SAA ELISA

Using samples of known SAA concentration provided with the ELISA kit, a standard curve was produced and a linear relationship established between OD and SAA concentration. The R^2 value for this curve was 0.963, demonstrating an excellent degree of correlation for the assay. The results from the 3 separate sera sets (described in Chapter 2, section 2.2.3.6 as TC1, TC2 and TC3) from sheep experimentally infested with *P. ovis* over a 6 week time course demonstrated increases of over 200-fold in the level of circulating SAA as disease progressed, with detectable differences in SAA by 4 weeks post-infestation (Figure 4.8).

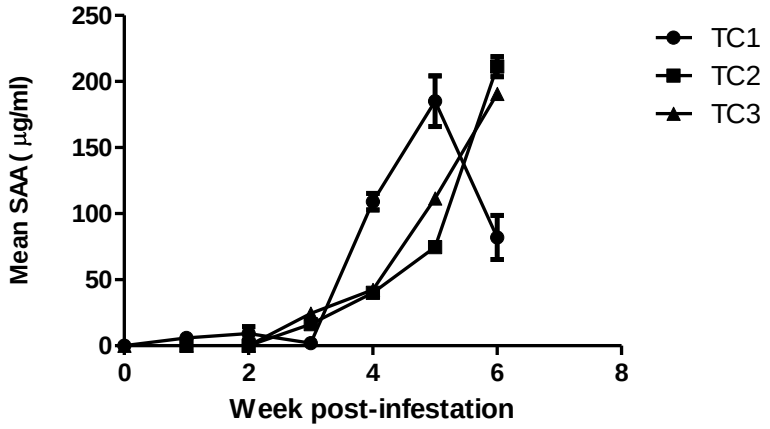


Figure 4.8: Mean SAA concentrations (±SE) as determined by SAA ELISA (Tridelta Development Ltd) in sera from sheep (TC1 n=6; TC2 n = 8; TC3 n = 6) experimentally infested with *P. ovis* over three separate 6 week time course trials. Samples were analysed for individual animals and tested in duplicate. Sera were diluted 1:500 in diluent buffer (supplied with the ELISA kit) unless SAA concentration was >300ng/ml and thereafter it was diluted 1:2000.

Mean serum SAA concentrations continued to increase between weeks 3 and 6, with the exception of the TC1 trial sera where SAA concentration at 6 weeks p.i had fallen to 81.99±16.597 μg/ml. This may be explained in this trial (TC1) as three out of the six sheep had naturally resolving lesions by week 6 post-infestation (Chapter 2, Table 2.1). Statistical analysis of the results depicted in Figure 4.8 was performed with a one-way ANOVA (Graph Pad Prism) and indicated that there were no significant differences in mean SAA serum concentrations until week 4 post-infestation in TC1 ($p \leq 0.001$) and by week 5 in TC2 ($p \leq 0.01$) and TC3 ($p \leq 0.01$).

The SAA ELISA results of the experimental trial TCT1 are shown in Figure 4.9 and tabulated in Table A4.4. SAA levels pre-infestation were 0.82±0.53 μg/ml and remained at this level over the first 3 weeks of primary infestation. By week 4 post-infestation, they had increased to 95.24±54.4 μg/ml and, by week 5, to 284.75±71.144 μg/ml which was the highest level recorded as, by week 6 post-infestation, there was a slight decrease in SAA levels to 217±46.36 μg/ml. Using a one-way ANOVA with Tukey's multiple comparison post-hoc test, the changes in SAA levels between time points were analysed and these data are shown on Figure 4.9. The increase in serum SAA levels between weeks 3 and 4 was not statistically significant, however between weeks 3 and 5 post-infestation it was highly significant ($p \leq 0.001$). Post-treatment, serum SAA levels fell within 3 days of treatment to 57.282±34.750 μg/ml ($p \leq 0.05$) and continued to decrease reaching pre-infestation levels within 10 days post-treatment. The secondary infestation was characterised by a rapid increase in SAA concentration of the same magnitude of the primary infestation peak, but within 24 hours post-secondary infestation. Within 2 weeks of

infestation, however, SAA levels were 2.256±2.464 µg/ml and they remained at this level until the end of the trial.

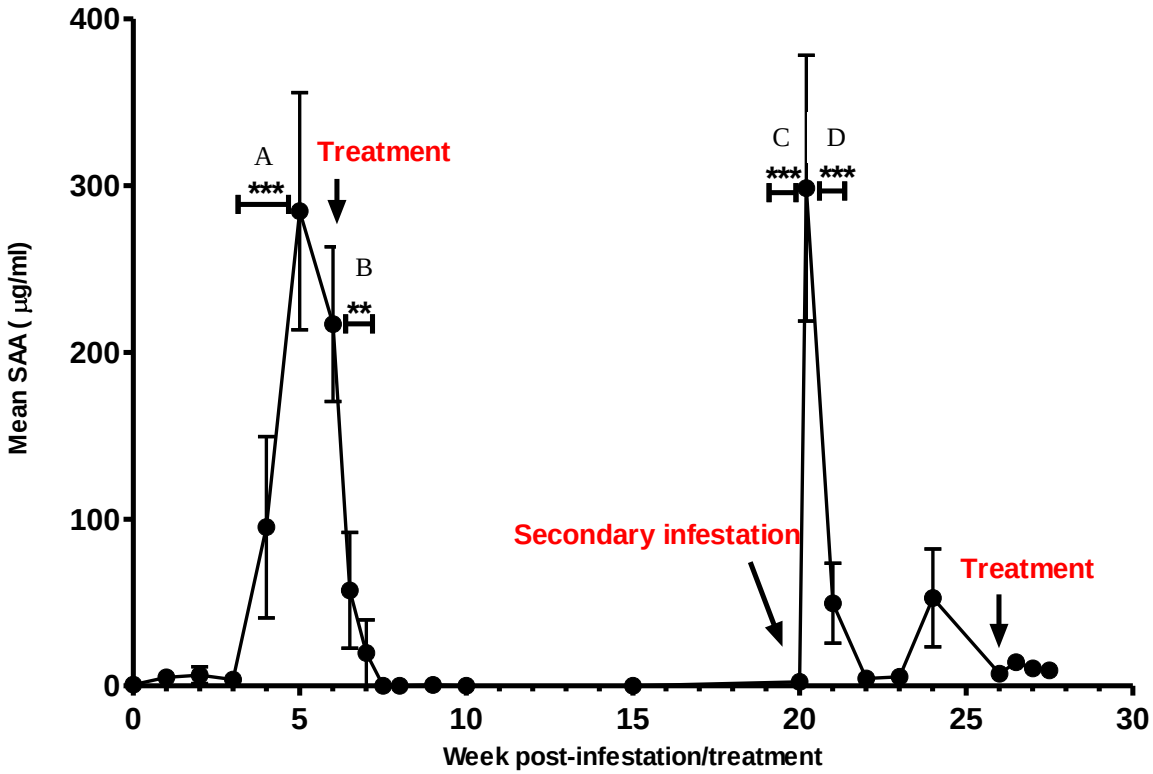


Figure 4.9: Mean SAA concentrations (± SE) through the time course of primary infestation with *P. ovis* for 6 weeks, and post-treatment over 14 weeks, followed by a further 6 week infestation and post-treatment for 2.5 weeks. Sera samples (n = 12) from individual sheep in the experimental trial TCT1 (described in A3.1.11) were analysed in duplicate by SAA ELISA (Tridelta Development Ltd). Bar A denotes a significant difference between weeks 4 and 5 post-infestation and bar B between point of treatment and 3 days post-treatment (primary infestation). Bar C denotes a significant difference between point of infestation and 24 hours post-infestation and bar D between 24 hours and 1 week post-infestation (secondary infestation). ****** $p \leq 0.01$; *******$p \leq 0.001$ as determined by one-way ANOVA (Graph Pad Prism).

Further analyses, as described in section 4.2.6, were used to define the changes in SAA levels following *P. ovis* infestation and treatment, and are presented in Table 4.3. The proportion of animals showing elevated serum SAA increased between weeks 4 and 5 post-infestation and all except one animal had returned to normal by 7 days post-treatment. The half life of SAA post-treatment was estimated at 0.84 days (95% CI = 0.73-0.99). The optimised range below which an animal was considered "normal" or above it "elevated" was 5.5-29.5µg/ml for SAA and a cut-off value of 29.5µg/ml was selected to optimise assay specificity. This cut-off value provided assay sensitivity of 1.0 and a specificity of 1.0, equating to a test accuracy of 100%.

Table 4.3: Distribution of animals (n = 12) following statistical analysis using the R statistical package (R Core Team, 2012) (described in section 4.2.6) into normal and elevated groups for SAA sera concentration across the time course of primary infestation and treatment (TCT1). *All sheep were treated with an injectable endectocide at week 6 p.i.

Week	0	1	2	3	4	5	6*	6.5	7	7.5	8
Normal	12	9	10	11	4	0	0	7	11	12	12
Elevated	0	3	2	1	8	12	12	5	1	0	0

The numbers of sheep which it would be considered necessary to test, based on the above analysis, in selected flock sizes and levels of disease prevalence are shown in Table 4.4. The trends in results were as for Hp, i.e. at higher disease prevalence fewer animals required testing and the probability of obtaining false positive results

in sheep scab negative flocks decreased. However, as the SAA assay showed higher sensitivity than the Hp assay, the probability that a negative flock would test positive at a given level of disease prevalence and test accuracy, was lower for SAA than Hp.

Table 4.4: Statistical analysis of SAA results to indicate the number of sheep in a flock which would require testing at selected levels of disease prevalence. This analysis was carried out using R statistical package (R Core Team, 2012) using previously defined test values of sensitivity (1) and specificity (1). Sera SAA results from the TCT1 primary infestation and treatment trial were used in this analysis. *Level of accuracy refers to the true detection rate in a positive flock. ** Probability that a negative flock will test positive.

FLOCK SIZE	DISEASE PREVALENCE (%)	*TEST ACCURACY REQUIRED	NO. ANIMALS REQUIRED TO TEST	**PROBABILITY OF DETECTION IN NEGATIVE FLOCKS
20	1	0.95	**19**	0.33
	5		**18**	0.32
	10		**17**	0.3
	20		**12**	0.22
50	1	0.95	**44**	0.6
	5		**35**	0.52
	10		**24**	0.4
	20		**13**	0.24
100	1	0.95	**70**	0.77
	5		**42**	0.59
	10		**25**	0.41
	20		**13**	0.24
200	1	0.95	**90**	0.85
	5		**43**	0.6
	10		**25**	0.41
	20		**13**	0.24

4.3.3 Alpha-1 acid glycoprotein

The results of the Western blot analysis of AGP in sera from sheep infested with *P. ovis* across a time course of infestation (Figure 4.10) showed bands at ~24 kDa, the

estimated MW of AGP, which increased in density particularly by weeks 5 and 6 post-infestation. However, the band density in lane 2 which contained the pre-infestation sera was also high, suggesting that AGP could be circulating at higher levels than optimal for a potential BM. The negative control (lane 6) suggested that the secondary antibody was not cross-reacting with IgG in the ovine sera.

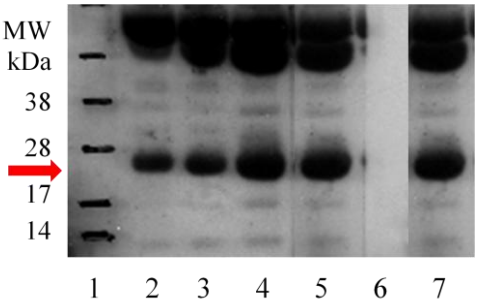


Figure 4.10: X-ray image of Western blot using pooled sera from sheep (n = 6) infested with *P. ovis* over a 6 week time course (TC1). The primary antibody was a monoclonal raised in mice against bovine AGP (Abcam) and used at a dilution of 1:500. The band of interest at 24 kDa is marked with a red arrow. Secondary antibody conjugate was rabbit anti-mouse HRP (Dako) used at a dilution of 1:5000. Visualisation was by ECL Plus (GE Healthcare) and X-ray development. Lane 1 = SeeBlue Marker (Invitrogen); Lane 2 = pre-infestation sera; Lane 3 = week 1 p.i sera; Lane 4 = week 3 p.i sera; Lane 5 = week 6 p.i sera; Lane 6 = -ve (no primary antibody) control; Lane 7 = +ve (ovine IgG) control using pooled 6 wk p.i sera (as in lane 5) probed with rabbit anti-ovine IgG HRP (Dako).

4.3.4 Assessment of serum SAA and Hp concentrations during other common diseases and conditions of sheep

As APPs are known to be non-specific indicators of inflammatory disease, serum Hp and SAA levels were measured by colorimetric analysis or ELISA, respectively, from serum samples taken during other common conditions of sheep. The serum Hp and SAA levels measured during most common sheep infections were of the same magnitude as those measured in sheep prior to infestation with *P. ovis* (Table 4.5).

Table 4.5: Mean serum Hp and SAA concentrations in other common sheep diseases and conditions. Hp (mg/ml ± SE) measured by colorimetric assay (ReactivLab) and SAA (µg/ml ± SE) assessed by SAA ELISA (Tridelta Development Ltd). All samples were assayed for individual animals in duplicate. Early gestation was 1 week post-conception and late gestation was 1 week prior to expected lambing date. *species included *T. circumcincta* and *N. battus.*

Infection	Mean Hp (mg/ml)±SE	Mean SAA (µg/ml)±SE
Early gestation	0.33±0.008	9.34±2.136
Late gestation	0.52±0.083	17.089±7.774
Gastro-intestinal nematodes*	0.04±0.034	0.297±0.007
Liver Fluke (*Fasciola hepatica*)	2.85±0.024	0.223±0.002
Sucking lice (*Linognathus spp.*)	0.52±0.508	68.47±22.325
Chewing lice (*Bovicola ovis*)	0.65±0.313	193.356±135.807
Orf	0.21±0.009	0.930±0.526
Johnes disease	2.25±0.344	21.433±10.471

For example Hp and SAA levels during experimental gastro-intestinal worm infections were 0.04±0.034 mg/ml and 0.297±0.007 μg/ml respectively. The exceptions to this were during liver fluke infection and Johnes disease, where Hp levels were 2.85±0.024 mg/ml and 2.25±0.344 mg/ml respectively, which is of the same magnitude as those measured 4-5 weeks post-infestation with *P. ovis.* SAA levels in chewing and sucking lice also showed the same magnitudes as 4-5 weeks post-infestation with *P. ovis* at 193.356±135.807 μg/ml and 68.47±22.325 μg/ml respectively, but Hp levels in both chewing and sucking lice were low.

4.3.5 Assessment of APP levels in clinically positive sheep during a field outbreak of sheep scab

As described in section 4.2.1.1, a natural field outbreak of sheep scab occurred at the MRI Firth Mains Farm (Burgess et al., 2012b) and samples from 12 sheep at three time points were available. These were pre-infestation, at the point of clinical diagnosis (two months later) and two months after treatment. Samples from the sheep identified as clinically positive were used to assess the levels of Hp and SAA in a field outbreak situation and were analysed using the assays as previously described in sections 4.2.2.4 and 4.2.3.

4.3.5.1 Haptoglobin

The results of the Hp assay for the 12 clinically positive sheep in the field outbreak of *P. ovis* are shown in Figure 4.11 and Table A4.5. The pre-infestation levels of Hp at 0.228±0.219 mg/ml were comparable to those pre-infestation in the TCT1 experimental trial (0.295±0.060 mg/ml) although the between animal variation was greater in the results from the natural outbreak samples when compared to the

experimental trial samples. Also lower levels of Hp during infestation and higher variation in sera Hp concentration between animals were obtained in the results from the natural outbreak samples compared with the experimental trial summarised in Figure 4.6.

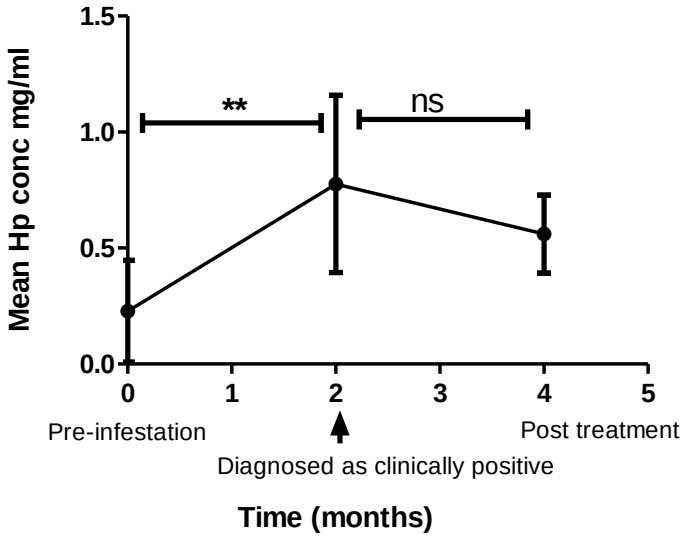


Figure 4.11: Mean serum Hp concentration (± SE) from sheep (n = 12) pre-infestation, at clinical diagnosis as positive for sheep scab and 2 months post-treatment in a field outbreak of sheep scab. Hp measurement by commercial assay (ReactivLab); sera from individual sheep tested in duplicate. **$p \leq 0.01$ as determined by a one-way ANOVA (Graph Pad Prism).

In the natural outbreak results, the increase in Hp from pre-infestation to the point of clinical diagnosis was statistically significant ($p \leq 0.01$) but the decrease in Hp levels 2 months post-treatment was not significant. The low mean Hp at the point of diagnosis, compared to *P. ovis* infested sheep in experimental trials, may have been due to a large proportion of the clinically positive sheep having low clinical scores,

i.e. they had very small lesions as it was early in the infestation. The data for the clinical scoring is described in the published paper (Burgess et al., 2012b)

4.3.5.2 Serum Amyloid A

The mean serum SAA levels of the clinically positive field infestation sheep are shown in Figure 4.12 and Table A4.6. The pre-infestation levels of SAA (1.068±0.237 μg/ml) in the field samples were higher than those in the TCT1 experimental trial (0.414±0.264 μg/ml).

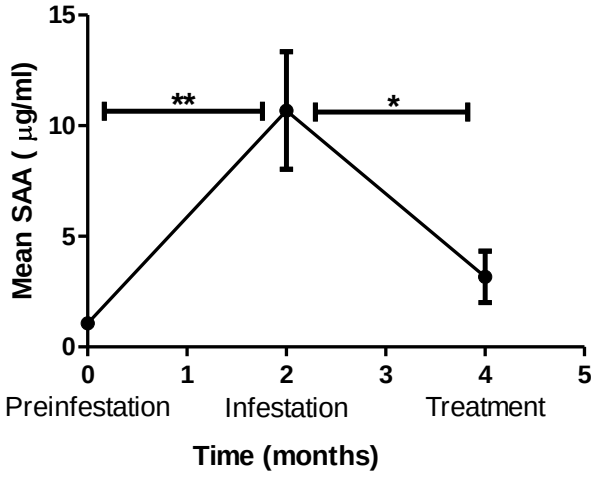


Figure 4.12: Mean serum SAA concentration (± SE) from sheep (n = 12) pre-infestation, at clinical diagnosis as positive for sheep scab and 2 months post-treatment in a field outbreak of sheep scab. SAA measurement by commercial ELISA (Tridelta Development Ltd); sera from individual sheep diluted 1:50 and assayed in duplicate. **p≤0.01; *p≤0.05 as determined by a one-way ANOVA (Graph Pad Prism).

At point of clinical diagnosis the recorded mean SAA (10.686±2.659 μg/ml) was lower than those during weeks 4, 5 and 6 p.i of the experimental trial TCT1, but

higher than those up to week 4 p.i (Table A4.4). This would have been expected as the lesions in the field outbreak sheep were small compared to those of the experimental trial sheep. In the field results, the increase in SAA from pre-infestation to the point of clinical diagnosis was statistically significant ($p \leq 0.01$) and unlike Hp, the decrease in SAA post-treatment was also statistically significant ($p \leq 0.05$) possibly due to the lower between animal variation in SAA when compared to Hp.

4.3.6 ELISA measurement of serum anti-Pso o 2 (specific mite antigen) IgG levels in sheep following infestation with *P. ovis*

The results of the ELISA analysis to determine the serum anti-Pso o 2 IgG responses in trial TCT1 are summarised in Figure 4.13 and in Table A4.7. By week 3 post-primary infestation the mean OD_{450} had increased from 0.573±0.019 pre-infestation to 1.197±0.115 ($p \leq 0.05$) and continued to increase until week 6 post-primary infestation. After treatment, the mean OD_{450} decreased from a 2 weeks post-treatment value of 2.663±0.066 to 1.727±0.098 by 3 weeks post-treatment ($p \leq 0.001$). After this the OD_{450} values continued to decline slowly and did not return to pre-infestation levels until the start of the secondary infestation trial, which was 14 weeks after treatment of the primary infestation. Applying the statistical analysis using the R statistical package (as described in section 4.2.6) to define the response of ovine antibody raised against Pso o 2, illustrated that the antigen-specific IgG level increased, exponentially, over time during the primary infestation period up to the point of treatment by 0.034 OD_{450} units per day (95% CI = 0.032-0.037). Following treatment the antibody response to Pso o 2 showed an exponential decay rate of 0.012 OD_{450} units per day (95% CI = 0.011-0.014) and the half life of the

antibody levels to Pso o 2 was estimated as being 56 days after treatment (95% CI 49 – 65 days).

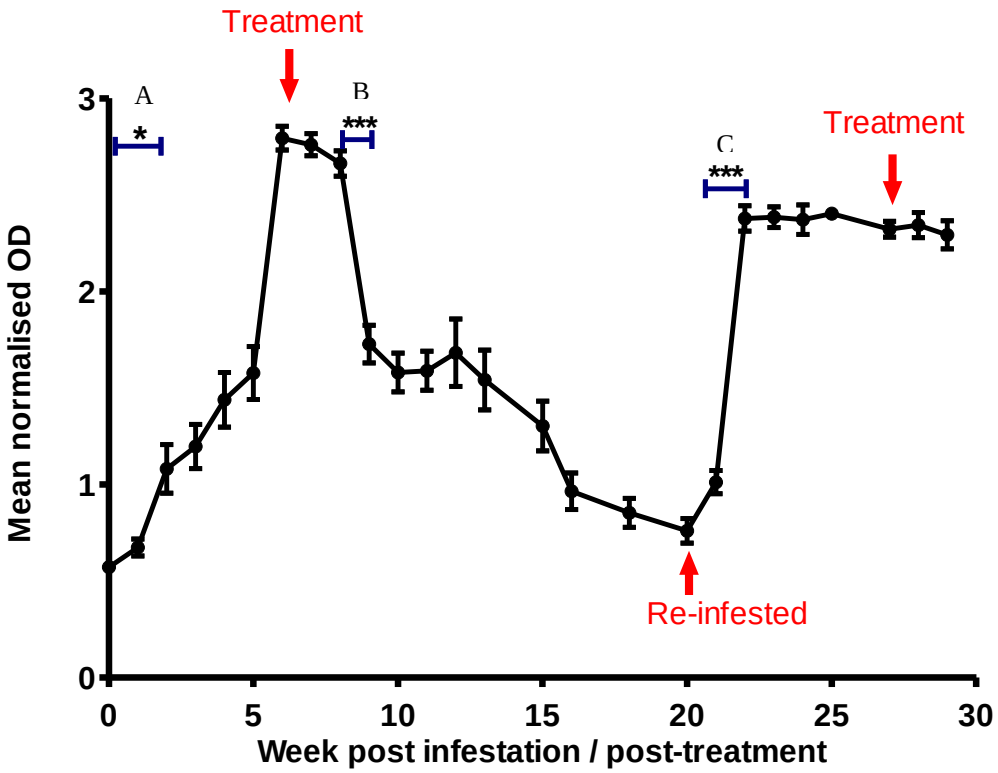


Figure 4.13: Mean normalised OD_{450} values (±SE) from ELISA to measure the ovine antibody response to Pso o 2 (a mite antigen) across the time course of primary infestation and then post-treatment using sera from sheep (n = 12) infested with *P. ovis* and treated with an injectable endectocide, followed by a secondary infestation and subsequent treatment (TCT1 trial). Coating antigen used was a recombinant Pso o 2 (rPso o 2) at 75µg/ml; conjugate used was a rabbit anti-sheep IgG HRP (Dako) at a dilution of 1:2000. Bar A denotes a significant difference between weeks 0 and 2 post-infestation; bar B between 3 and 4 weeks post-treatment (primary infestation) and bar C between 24 hours and 1 week post-infestation (secondary infestation). *p≤0.05; ***p≤0.001 as determined by a one-way ANOVA (Graph Pad Prism).

Positive inter-plate controls had previously been performed for this ELISA using HI sera analysed over 5 plates giving a mean OD of 2.5 (Nunn et al., 2011). The results obtained here were normalised by multiplying the mean sample OD by 2.5 and dividing by the mean positive control OD obtained for that plate.

By week 2 post-infestation 8 out of 12 animals (Table 4.6) showed an IgG response to Pso o 2 and more than half the animals continued to show elevated antibody levels to Pso o 2 at 19 weeks post-treatment. As the anti-Pso o 2 antibody levels increased at approximately three times the rate at which they decreased, it is evident that antibody levels were not an accurate measure of current disease status post-treatment, but were effective for the early diagnosis of sheep scab during this trial.

Table 4.6: Distribution of animals (n = 12) after statistical analysis using the R statistical package (R Core Team, 2012) (described in section 4.2.6) into normal and elevated groups for host antibody response to Pso o 2 across a time course of primary infestation with *P. ovis* and subsequent treatment (TCT1). * All animals were treated with injectable endectocide (Dectomax, Pfizer Animal Health) at week 6 post-infestation.

Week	0	1	2	3	4	5	6*	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Normal	12	9	4	3	1	1	0	0	0	0	0	0	0	1	1	1	2	4	5	5	6
Elevated	0	3	8	9	11	11	12	12	12	12	12	12	12	11	11	11	10	8	7	7	6

4.4 Discussion

Two fragments of Hp, present in the sera of sheep infested with *P. ovis* at week 5 post-infestation, were identified by MALDI analysis, following which Western blot analyses indicated a clear relationship between the band densities of Hp and SAA and lesion size; and the band densities of AGP over time during *P. ovis* infestation. Although the evaluation of AGP was preliminary, the results of Western blot analysis of AGP levels in the sera of *P. ovis* infested sheep indicated that this APP also had potential as a BM for sheep scab, although the differences between levels in pre-infestation and post-infestation sera may not be sufficient. AGP has previously been suggested as a potential marker for chronic conditions in sheep due to its low levels of <0.1 mg/ml in healthy, control sheep compared with a peak level of 0.4 mg/ml in the chronic phase of the disease (Eckersall et al., 2007) and the continued production of AGP during the transition from acute to chronic phase in CLA in sheep (Eckersall et al., 2007). This difference in the profile of AGP in comparison to Hp and SAA has also been found in cattle where it was shown to be useful in discriminating between acute and chronic conditions (Horadagoda et al., 1999) and in goats showing an inflammatory response due to subcutaneous injection of turpentine oil (Gonzalez et al., 2008). As sheep scab has a chronic phase of disease, following the initial acute inflammatory stage (Van den Broek and Huntley, 2003a), further work should include evaluation of AGP as a potential marker for *P. ovis* infestation and, as there is now a commercially-available ovine AGP assay (Tridelta Development Ltd), quantitative validation of AGP could be performed.

Following the promising preliminary Western blot analysis results for Hp and SAA, these APPs were quantified using commercially-available assays. This analyses

demonstrated that Hp and SAA increased in serum concentration as *P. ovis* infestation progressed showing statistically significant increases ($p \leq 0.001$) by week 4-5 post-infestation, confirming previous studies showing increases in serum Hp and SAA levels during a range of inflammatory diseases in ruminants (Heegaard et al., 2000, Petersen et al., 2004, Eckersall and Bell, 2010).

Considering first the magnitude of the increases in Hp and SAA serum concentrations in response to *P. ovis* infestation, the results obtained agreed with a previous study investigating the response of Hp and SAA in Alpine ibex (*Capra ibex*) to a natural outbreak of *S. scabiei* infestation (Rahman et al., 2010). Over an experimental six week time course of infestation with *P. ovis*, Hp levels in sheep serum increased by more than 10-fold (up to a maximum level of 3.53±0.89 mg/ml) compared with pre-infestation levels, whereas SAA levels increased approximately 350-fold in the same period to a maximum level of 284.75±71.14 μg/ml. These results demonstrated a similar trend to the levels of Hp and SAA measured in Alpine ibex infested with *S. scabiei* where mean Hp levels during infestation increased to 3.72±0.65 mg/ml compared with healthy controls (0.58±0.09 mg/ml) and 130.7±0.16 μg/ml and 8.7±0.13 μg/ml in infested and control animals respectively for SAA (Rahman et al., 2010). The increase in serum concentrations of Hp were of a similar magnitude in both studies, although the *S. scabiei* infestation was a natural outbreak whilst the *P. ovis* study used experimental infestations. This may be explained as the *S. scabiei* outbreak was well established and animals tested as positive for Sarcoptic mange exhibited clear clinical symptoms as determined by skin lesions (Rahman et al., 2010). Hp was concluded to be a minor APP in Alpine ibex by the authors due to

the smaller fold change increases when compared with SAA (Rahman et al., 2010) which may also be concluded when describing the increase in Hp levels in sheep responding to *P. ovis* infestation. Compared to the increase in Hp in both Alpine ibex and sheep during Sarcoptic and Psoroptic mange infestations respectively, the larger fold change increases measured for SAA would classify it as a major APP and the increase from baseline levels in many inflammatory diseases in ruminants has been reported as being generally higher for SAA than for Hp (Gabay and Kushner, 1999).

The levels of Hp measured in this study prior to exposure to *P. ovis* ranged from 0.29±0.06 to 0.61±0.19 mg/ml. APPs have been reported as being present at undetectable, to very low levels in the serum of healthy animals (Cray et al., 2009) but, recently, a study investigating the APP response to scrapie in sheep found large individual animal variation in Hp and SAA prior to clinical disease onset and suggested this may be due to underlying subclinical conditions (Meling et al., 2012). Previous work has indicated that Hp and SAA are non-specific in terms of inflammatory disease, injury or infection, but highly sensitive, effective markers of inflammation in ruminants (Petersen et al., 2004, Eckersall and Bell, 2010) suggesting that any inflammatory event in the animal may cause a temporary rise in Hp or SAA. In comparison to Hp prior to *P. ovis* infestation, serum SAA was measured at lower levels, for example, mean SAA concentration pre-infestation during the time course trial (TC1) was below the limits of detection compared with Hp which was 0.55±0.19mg/ml, suggesting that SAA may potentially be a more suitable BM to indicate current disease status during *P. ovis* infestation in sheep than Hp.

Compared to a previous study which investigated the Hp, SAA and AGP response in sheep to infection with bacteria causing CLA, the magnitude of increased levels of Hp and SAA to *P. ovis* infestation was larger. For example, peak Hp and SAA in sheep with CLA was 1.65±0.21 mg/ml and 18.1±5.2 μg/ml, respectively. However, these peaks were achieved by day 7 post-infection, which is considered to be the point at which the acute disease becomes a chronic infection (Eckersall et al., 2007), whereas in the APP response to *P. ovis* infestation, sera Hp concentration was still increasing at the point of treatment or slaughter (6 weeks p.i) with peak Hp levels of 3.53±0.64 mg/ml and peak SAA levels of 284.75±71.14 μg/ml. Hp and SAA have been previously reported to be measurable by day two post-infection in acute inflammatory diseases such as mastitis and metritis (Eckersall and Bell, 2010). In contrast to this, in the work presented herein, the proportion of animals with a *P. ovis*-induced elevation in APPs did not occur until weeks 4-5 post-infestation in the case of Hp (Table A4.1), or marginally earlier at week 4 for SAA (Table A4.3). A possible explanation for this is that disease initiation in sheep scab has been described as having a "lag phase" at the start of infestation and lasting for several weeks as the mites become established, after which the mite numbers increase exponentially (Bates, 1997). Lesion size development follows this pattern in mite numbers, as the increasing numbers of mites move out from the margins of the lesion onto healthy skin, as shown by the lesion size data recorded in the TCT1 trial (Figure 4.1).

In order to diagnose current disease status in sheep exposed to *P. ovis*, it is crucial that any potential BM declined rapidly upon treatment or disease resolution and this

study has demonstrated that Hp and SAA return to pre-infestation levels rapidly, with estimated half life of less than 3 days, illustrating the potential of including either or both of these APPs in an improved diagnostic test for sheep scab where current disease status is important. This is likely to be the case in Scotland due to the Sheep Scab 2010 Order, where confirmation of successful treatment is required before movement restrictions are removed from the affected farm. The legislation allows this to occur 16 days post-treatment when it is still extremely difficult to assess by clinical examination if any active infestation remains. However, if Hp and /or SAA were used as BMs of current disease status as demonstrated herein, they would have returned to baseline levels before 16 days indicating successful treatment.

Comparing the two APPs quantified here, SAA responded more rapidly to treatment of *P. ovis*-infested sheep than Hp, which was also observed in a previous study investigating the response of these APPs to CLA in sheep (Eckersall et al., 2007). The SAA response to *P. ovis* infestation was of a higher magnitude (350-fold increase compared to 10-fold increase for Hp) and more rapid in its initiation and in its resolution (as determined by the half life) than Hp which suggests that if a single APP BM was to be used for indicating current disease status, SAA would be the more suitable. In addition, the SAA response was a more accurate discriminatory indicator of current disease status in the TCT1 study than Hp, due to the higher sensitivity obtained for the optimised cut-off values which were estimated using the data from the primary infestation and post-treatment element of the TCT1 trial. Applying these cut-off values and the test sensitivities and specificities obtained, the SAA test resulted in fewer false positives in negative flocks, when compared to Hp

(see Tables 4.2 and 4.4). Minimising false positive results is recognised as a critical factor for a sheep scab diagnostic assay due to the problems associated with treating non-infested sheep in terms of cost, time and selecting for resistance (Burgess et al., 2012b). This is therefore an important consideration when comparing the accuracy of SAA and Hp as BMs to indicate current disease status during *P. ovis* infestation and after treatment.

When Hp and SAA levels were investigated during other common field diseases and conditions of sheep, they were predominantly found to be at levels equivalent to the sheep scab negative values, remaining below 0.6 mg/ml for Hp and 22 μg/ml for SAA. Importantly, as GINs commonly affect sheep with no clinical symptoms, Hp and SAA levels were not elevated during the GIN experimental infections tested here. Although Hp was elevated in liver fluke and Johnes disease and SAA was elevated in lice infestation in this analysis, it is evident from the standard errors in these results that there was large between animal variation and therefore these recorded measurements may be due to other underlying conditions. Analysis of additional sera samples from sheep infected with these diseases would be required to confirm serum levels of Hp and SAA during these infections. In addition, when one of these APPs was high in a particular infection, the other was low. This illustrates the potential for using a signature of BMs rather than an individual BM as a method of combating lack of specificity of the APPs and will be further discussed in the general discussion in Chapter 5.

Hp and SAA, although showing potential in validation using sera from animals infested experimentally with *P. ovis*, require further validation using field samples

from sheep scab positive and negative animals. The 12 clinically positive sheep from the natural sheep scab outbreak analysed herein, showed much lower mean Hp and SAA levels at the point of diagnosis (see Figures 4.11 and 4.12) and greater between animal variation than those from the experimentally infested sheep. This may be explained as the field outbreak at the point of clinical diagnosis was in its early stages with most of the diagnosed clinically positive animals having low clinical scores and small lesions (Burgess et al., 2012b). Further validation of Hp and SAA as potential BMs for current disease status in sheep will therefore be required using sera from sheep confirmed positive and negative in natural outbreaks.

Compared to Hp and SAA levels, the development of ovine IgG to the mite antigen Pso o 2 provided earlier diagnosis of *P. ovis* infestation as shown in Table 4.6, where the majority of animals showed an elevated serum IgG response to Pso o 2 in the primary infestation by week 2 post-infestation. This agreed with previously reported results using this assay (Nunn et al., 2011). As previously discussed, the problem with the existing assay in diagnosing current disease status after treatment or on disease resolution is due to the persistence of the antibody. When the decreases in Hp and SAA levels post-treatment were compared to those of the Pso o 2 specific IgG levels, it was evident that Hp and SAA levels gave a rapid indication of current disease status post-treatment, whereas the antibody response was still measurable 20 weeks post-treatment. The differences in half life also illustrated the differing responses to treatment i.e. 56 days for the antibody response, compared to 2.3 days for Hp and 0.84 days for SAA. However, as serum Hp and SAA levels increase in many inflammatory diseases in ruminants, these APPs could not be used alone as

BMs for the diagnosis of sheep scab. The relative merits of the tests measuring IgG response compared with Hp/SAA levels indicate that a combined diagnostic test incorporating the IgG response to Pso o 2 along with Hp and/or SAA would result in a highly specific test which would indicate early infestation and current disease status after treatment.

4.5 Conclusions

- Hp and SAA, two major APPs of ruminants, were evaluated as potential BMs for use in the diagnosis of active sheep scab infestation.
- Immunoblotting indicated a positive relationship between Hp and SAA sera concentration and disease progression, as indicated by lesion size development.
- Quantitative analysis showed that Hp and SAA levels in sheep sera increased following infestation with *P. ovis* but were not statistically significantly until 4 weeks post-infestation.
- SAA was the more discriminatory marker, with lower pre-infestation levels and higher sensitivity at the optimum cut-off values than Hp.
- After treatment of infested sheep with an acaricide, Hp and SAA levels in sheep sera declined rapidly, with a half-life of less than 3 days.
- In comparison, the half life of Pso o 2-specific IgG in sheep sera following acaricidal treatment was estimated as being 56 days.
- Further evaluation of Hp and SAA as BMs indicating current disease status during sheep scab outbreaks is required using extensive field positive and negative samples to optimise the assays.

Chapter 5: General Discussion

Sheep scab, a disease caused by the ectoparasitic mite *P. ovis*, is endemic in the national flock and is a major welfare and production issue for UK sheep farmers (Van den Broek and Huntley, 2003a) and worldwide. The problems associated with disease control have recently been tackled in Scotland through the Sheep Scab (Scotland) Order 2010 in which the disease was made notifiable, and therefore accurate diagnosis of active infestation is crucial. As existing methods of diagnosis are unreliable, particularly in early infestation where the disease can be sub-clinical (Bates, 1999c), a new diagnostic assay based on the host antibody response to a mite antigen, Pso o 2, has been developed. This assay is highly sensitive and specific and can detect disease from two weeks post-infestation (Nunn et al., 2011). However, as the Pso o 2-specific antibody is detectable in serum for several months after successful treatment (Burgess et al., 2012b), this test does not indicate current disease status post-treatment or following active disease resolution.

This thesis aimed to address this issue by identifying and evaluating potential BMs for sheep scab which indicate current disease status by increasing in serum concentration during early infestation, from low pre-infestation levels, and returning to low levels rapidly following successful treatment. Overall, 178 potential BMs were identified, 13 preliminarily evaluated and three selected for further quantitative evaluation.

To enable selection of the most suitable BMs, a bioinformatic analysis was initially performed which resulted in the identification of a list of 178 genes differentially expressed in circulating leukocytes across a time course of *P. ovis* infestation

(Burgess et al., 2012a) ranked according to set criteria. It is widely recognised that the approach used in this study, using transcriptomics to anticipate changes in plasma proteins levels between health and disease states has limitations, but as ruminant - specific protein arrays and the technology to accurately analyse low abundance proteins in sera are not yet available, genomic approaches are currently commonly adopted (Kulasingam et al., 2010).

A recent study has approached the search for potential diagnostic BMs for bovine tuberculosis (bTb) using similar methods i.e. transcriptional gene profiling and bioinformatic analysis of microarray results (Aranday-Cortes et al., 2012). This bTb study initially used a murine bTb model to identify promising candidates post-infection from microarray analysis of RNA from *in vitro*-stimulated splenocytes and lung cells, to identify possible BM candidates and guide the selection of such markers in cattle. Genes with the largest fold-change increase between healthy and *Mycobacterium bovis*- infected mice were selected and their bovine orthologues were evaluated by real-time PCR (qPCR) using RNA extracted from peripheral blood mononuclear cells (PBMCs) from uninfected and naturally infected cattle. Five out of the 14 up-regulated genes based on the murine transcriptomic analysis were also significantly up-regulated in bovine PBMCs during bTb infection and included interferon gamma, interleukin-22, interleukin-17A and chemokine ligand 9 and 10 (Aranday-Cortes et al., 2012). BMs for bTb, defined in the murine system, have therefore been shown to guide selection of BMs in cattle, providing a more cost effective model with a wider variety of available immunological reagents available compared with a ruminant model. Unfortunately, there is no such available model for

sheep scab as, although the rabbit mite, *P. cuniculi,* is thought to be a rabbit-adapted con-specific variant of *P. ovis* (Pegler et al., 2005), the differences in pathology between *P. ovis / P. cuniculi* in rabbits compared with sheep (Rafferty and Gray, 1987) indicate the rabbit would not be a suitable model for sheep scab. Following the genomic analysis in the bTb study, confirmation will be required that there is increased production of the identified potential BMs at the protein level to enable developing this research into a practical antibody-based diagnostic test for bTb (Aranday-Cortes et al., 2012).

In applied BM research, the lack of analyte-specific reagents, essential for BM validation using techniques such as ELISA, was the major reason suggested for the bottleneck from BM discovery to clinical translation (Pepe et al., 2008). This was also an issue for the preliminary evaluation of the ranked list of potential BMs for sheep scab after the bioinformatic analysis, as there were no commercially-available antibodies specific to the ovine proteins for any of the highly ranked BMs at the time of testing. As noted previously, this is a recognised problem in veterinary immunology research which is now being addressed (Entrican et al., 2009) and because of work such as the Veterinary Immunology Committee Toolkit (Entrican and Lunney, 2012) recent increases in veterinary species specific assays, such as diagnostic applications of major APP testing in companion animals, are being developed (Kjelgaard-Hansen and Jacobsen, 2011). In the future this work should also lead to further assays being commercialised for routine health and welfare tests in ruminant veterinary medicine and, as such, the list of potential BMs for sheep scab produced in Chapter 2 is a useful resource for future BM evaluation.

The problem of a lack of species specific analytes in ruminant immunology was illustrated by the absence of, or weak, antibody/antigen recognition obtained in many of the Western blot analysis results (Chapter 2), even when relatively high homology was found between the ovine/bovine and human amino acid sequences for the protein under investigation, such as that seen with collectin 11 and oxytocin (Chapter 2, Table 2.5). Despite these problems, the results obtained in the preliminary evaluation of C4BPB suggested that it was a potential BM for *P. ovis* infestation in sheep and as such warranted further investigation.

The ovine *C4BPB* gene was sequenced (Chapter 3) and a recombinant protein was expressed successfully in a bacterial system. The recombinant protein obtained was insoluble in an aqueous solution suggesting it would be advantageous to use a mammalian system for future expression of ovine C4BPB, as the method of choice for expressing sheep proteins, e.g. see Demain and Vaishnav, 2009. Optimisation of the ELISA developed in Chapter 3 to quantify C4BPB in sheep serum resulted in a sandwich ELISA which, when used to analyse C4BPB concentrations in ovine sera showed promising preliminary results. C4BPB levels in the sera of sheep prior to infestation with *P. ovis* were low with statistically significant increases in mean OD_{450} by three weeks post-infestation. Although this was one week later than the measurable increase in host antibody response to Pso o 2 (Nunn et al., 2011), the decrease in C4BPB level in response to acaricide treatment was rapid, with significantly decreased OD_{450} values by day three post-treatment and serum C4BPB levels halved by one week post-treatment, illustrating the potential of C4BPB to indicate current disease status. In comparison, the decrease in host antibody levels to

Pso o 2, as measured by half-life, was 56 days. It was concluded that further validation of C4BPB as a BM would be required, particularly using samples obtained from natural outbreaks.

One issue with the sandwich ELISA developed in Chapter 3 was that it included an expensive, anti-human mC4BPB capture antibody, which, if used for the high numbers of samples required to validate a diagnostic test, would be cost prohibitive. Further work would therefore be required to develop an alternative capture antibody, possibly by production of a mouse anti-ovine C4BPB monoclonal antibody. This may also improve the assay as the capture antibody would be a species specific one, potentially leading to a stronger signal.

An alternative or additional approach is to use existing BM assays, and, in Chapter 4 evaluation of the major ruminant APPs, SAA and Hp was performed using commercially available diagnostic assays which confirmed the potential of SAA and Hp as BMs for current disease status in sheep scab diagnosis. SAA serum concentration increased marginally earlier, by a higher magnitude and from lower pre-infestation levels than Hp and was the more accurate, discriminatory indicator of current disease status in the experimental trial studied. The serum samples from clinically positive sheep in a natural outbreak of sheep scab, which were analysed for Hp and SAA concentration, produced lower levels of these APPs compared to the experimental trial samples tested, which may have been a result of the natural outbreak being in early stages (as most of the sheep diagnosed as clinically positive had small lesions).

It is an important consideration that because of differences in the epidemiology of natural sheep scab outbreaks compared to experimental trials that the size and timing of the APP response will vary between them, mainly due to the lower mite numbers initiating the infestation in a natural outbreak and the longer time for the infestation to spread through a flock in the field.

The infestation of sheep by *P. ovis* has been described as following a characteristic cycle of 'lag', 'growth' and 'recovery' stages reflecting mite numbers and lesion size development (Bates, 1997) but a simulation model analysing the population dynamics of *P. ovis* infestation in naturally infested and one experimentally infested sheep suggested that the observed pattern of mite numbers and lesion growth was a result of an exponential increase in numbers with the initial 20 days of the exponential growth period equating to the previously described 'lag' phase (Wall et al., 1999). As the number of mites present at any time point during the infestation in its exponential growth phase depended on the number of mites starting the infestation (Wall et al., 1999), infestation dynamics are likely to vary between natural outbreaks and experimental trials and it is therefore crucial that further field-derived serum samples are analysed to optimise Hp and SAA cut-off values if they are to be used to assess current disease status in natural outbreaks of sheep scab.

This can be illustrated if the cut-off values, to differentiate between 'normal' and 'elevated' animals for APPs estimated on the experimental trial data are used on the results from the natural outbreak. For example, the cut-off value for Hp was 1.260 mg/ml and for SAA was 29.5 μg/ml, whereas the mean Hp level in the field positives was 0.776 mg/ml and for SAA was 10.686 μg/ml. The cut-off values used in the statistical analysis of the TCT1 trial results were set at a high level to minimise false

positive (FP) results. Previously it has been suggested that for an accurate diagnostic test for sheep scab high specificity is required as it is essential to minimise FP results and thereby eliminate unnecessary treatments as, for example, has been obtained in the current diagnostic ELISA (Burgess et al., 2012b). Reducing the cut-off values in the APP analysis to take the expected lower Hp and SAA levels in the serum of sheep naturally infested into account, will therefore reduce the accuracy of the test (Obuchowski, 2003).

It is evident that many more clinically positive and negative samples from natural outbreaks will need to be analysed so that optimal cut-off values for Hp and SAA can be set using ROC curves. These are plots of test sensitivity versus 1- specificity for all possible cut-off points, where the area under the ROC curve (AUC) is recognised as being a reliable measure of test accuracy as it does not depend on disease prevalence (Obuchowski, 2003). This is an important point when considering sheep scab diagnosis, as disease prevalence has previously been shown to be highly variable depending on factors such as sheep density, elevation, temperature and access to common grazing (Bisdorff et al., 2006) and has been observed to be persistent on particular farms or geographic areas suggesting likely management or environmental risk factors associated with these farms, allowing the development of risk models directing effective management towards sheep scab control (Rose et al., 2009). This model has been further refined as results have suggested upland and lowland sheep farms should be approached as two distinct epidemiological systems due to access to common grazing, and therefore direct contact with neighbouring sheep, in the former (Rose and Wall, 2012). With the evident differences in sheep scab epidemiology and disease prevalence depending on geographical area and sheep

farming systems, it will be important to incorporate ROC curve analysis into further BM test validation, and this should be carried out with C4BPB validation as well as for Hp and SAA. A solution to estimating cut-off values for use in the many different scenarios the BM test could be applied in, may be to have different cut-off values calculated depending on the situation for which the test is to be used in. For example, where some sheep are obviously pruritic in a flock the cut-off value could be decreased to increase the chance of getting all positives included (even sub-clinical animals). This would also be the case in a quarantine or eradication campaign, whereas in contrast to this, for random testing in a livestock market the cut-off value could be increased to increase the accuracy of the test as false positives would be highly undesirable in this situation.

Another method of increasing test accuracy may be to use a signature of BMs in the assay, rather than a single protein assay. This has previously been suggested due to the variation in response obtained from different APPs, particularly with reference to acute and chronic phases of inflammatory disease, as the use of a panel or signature of BMs may provide more powerful diagnostic information and increase assay specificity, compared to a single APP assay (Eckersall et al., 2007). Future work validating the use of a BM assay for *P. ovis* infestation should include AGP, if it was quantitatively evaluated as a potential BM, as it has a previously suggested role as a potential BM of chronic inflammatory conditions in sheep (Eckersall et al., 2007) The idea of using a signature of APPs as BMs for disease diagnosis has been practiced in human medicine by their inclusion as indices in general health profiles for several decades (Eckersall and Bell, 2010, Gruys et al., 2006). As these indices

have previously been shown to increase the sensitivity and specificity of APPs as clinical markers, a signature of positive APPs, such as Hp and SAA, could be incorporated with a negative APP, e.g. albumin, if validated successfully, with the aim of combining them into a multiplex BM test to assess if the diagnostic capability of the test in indicating current disease status in sheep scab outbreaks was improved. The promising results obtained using C4BPB as a potential BM for sheep scab indicates that, if a signature of BMs increased the sensitivity and specificity of the diagnostic test, a combination of C4BPB, SAA and/or Hp should be investigated. Preliminary results, using the ELISA developed to detect C4BPB in sheep sera, demonstrated that serum C4BPB levels are elevated earlier than SAA or Hp during *P. ovis* infestation and decrease as rapidly as SAA following treatment. Using all three evaluated BMs would increase the specificity of the assay as, for example, Hp and SAA reacted to different field infections in sheep but did not react to the same infections; C4BPB increased in serum concentration earlier post-infestation than either Hp or SAA and all three decreased quickly enough post-treatment to be useful in the context of the Sheep Scab (Scotland) Order 2010. Positive diagnosis of sheep scab on Scottish farms results in movement restrictions for 16 days post-treatment until the sheep can be confirmed as having been successfully treated. If the BM assay was used in this situation, movement restrictions could potentially be shortened as sheep may be confirmed as successfully treated three days post-treatment.

Finally, the potential of the above signature of BMs should be assessed in combination with the existing Pso o 2 ELISA. As previously discussed, this ELISA provides a highly sensitive and specific diagnosis of early *P. ovis* infestation [prior to

obvious clinical symptoms (Burgess et al., 2012b)] and is currently being commercialised as a diagnostic ELISA, with further development ongoing to investigate possible production of a lateral flow assay (LFA) for pen-side diagnosis. To solve the problem of the existing assay in its inability to diagnose current disease status post-treatment, the BMs evaluated in this study could be multiplexed with the Pso o 2 ELISA, providing an improved diagnostic test which would provide a very useful tool for the eradication or control of sheep scab.

A pen-side test for the early diagnosis of sheep scab would be of particular benefit to the sheep industry; as such a test is likely to be useful on farm to confirm active infestation or successful treatment, or at livestock markets and agricultural shows. ELISAs enable highly sensitive, quantitative and multiplexed measurements but may require multiple steps (Lequin, 2005) and costly laboratory equipment so are therefore unsuitable for use in the field. In contrast, LFAs are ideal for pen-side testing as they are relatively inexpensive, portable and give rapid results without the need for a laboratory. To date however, they are less sensitive than ELISA and are unable to provide quantitative measurements and are therefore used in situations requiring a single YES/NO result such as human pregnancy tests (Posthuma-Trumpie et al., 2009). Recent advances in microfluidics technology have potential to offer rapid, reliable detection of multiple analytes from a single biological sample using portable, low-cost devices (Yager et al., 2006, Whitesides, 2006). The main advantage of the microfluidic chip is its ability to perform diagnostic processes without the need for skilled operators or a well equipped laboratory and, as such, devices have now been developed to perform multiplexed bioassays using low-cost paper materials for inexpensive point of care analysis in human medicine (Martinez

et al., 2007). This development may have potential for use in developing countries where ease of storage (no refrigeration required), ease of use and low cost are highly advantageous. Parallels may be drawn here to the UK sheep industry where cost margins are tight, particularly in the upland sector, therefore lower costs are beneficial, as are ease of use and storage for tests which are aimed to be carried out pen-side, in a variety of difficult conditions.

As current methods for testing APP levels are limited to laboratory-based tests which are costly and require separate tests for each factor, the development of a paper-based microfluidics platform for pen-side testing would be highly beneficial. The simultaneous assessment of BMs combined with the measurement of antibody levels specific for mite Pso o 2 on a microfluidics platform would provide an accurate, rapid, cost-effective and early diagnostic test indicating current disease status, which would be a powerful tool for use in sheep scab disease control or eradication schemes.

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Appendix

A2.1 Buffer Preparation

A2.1.1 MES buffer for electrophoresis

A litre of running buffer was made up by mixing 50ml NuPage MES SDS Running Buffer (20X) (Invitrogen) with 950ml ultrapure (UP) water.

A2.1.2 Wash buffer and antibody diluent

A litre of wash buffer/antibody diluent was obtained by mixing 100ml 10xPBS with 900ml UP H_2O, 29.22g NaCl and 5ml Tween 80 (Sigma-Aldrich).

Table A2.1: Secondary antibodies used for the evaluation of potential BMs by Western blot analysis, including optimal concentrations used as determined by titration blots. All secondary antibodies were HRP conjugated except *where no secondary antibody was required as the primary antibody was HRP conjugated.

Candidate protein	Secondary antibody	Source and catalogue number	Concentration
Osteopontin (SPP)	Pig anti-rabbit	Dako P0399	1:2000
Calgranulin (SA100A9)	Pig anti-rabbit	Dako P0399	1:2000
Calgranulin (SA100A8)	Rabbit anti-mouse	Dako P0260	1:1000
Complement 4 binding protein (C4BPB)	Rabbit anti-mouse	Dako P0260	1:1000
Tumour necrosis factor (TNF-α)	Streptavidin	Dako P0397	1:2000
Oxytocin (OXT)	Goat anti-guinea pig	Abcam ab6908	1:2000
Potassium voltage gated channel (KNCQ1)	Chicken anti-rabbit	Abcam ab6829	1:3000
Solute carrier family 7 (SCL7A11)	No secondary required*		
Collectin11 (COLEC11)	Pig anti-rabbit	Dako P0399	1:2000
Tenascin C (TNC)	Rabbit anti-chicken	Sigma A9046	1:30000

1. SPP: Human (accession no P10451) and ovine (Q9XSY9)

```
MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLLAPQNA  Human
MRIAVICFCLLGIASALPVKPTSSGSSEEKQLNNKYPDAVATWLKPDPSQKQTFLEPQNS  Ovine
*************:.*:*** :.********* ***********:*******.:* ***:

VSSEETNDFKQETLPSKSNESHDHMDDMDDEDDDDHVDSQDSIDSNDSDDVDDTDDSHQS
VSSEETDDNKQNTLPSKSNESPEQTDDLDDDDENS-----QEVNSDDSDDAETPDDSDHS
******:* **:********* :: **:**:*::.     :.::*:****.:  ***.:*


DESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLRSKSKKFRRPDIQ
NESHHSDESDE--ADFPTDIPTIAVFTPPFPTESTNDGRGDSVAYGLKSKSKKFRRSNVE
:**********  :*****:*:  **** .** .* *******.***:******** :::

YPDATDEDITSHMESEELNGAYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSH
SPDATEEDFTSHIESEEMHDAPKKTSQ----------------------LTDHSEETNSD
****:**:***:****:. * *                          * *:* **.*.

KQSRLYKRKANDESNEHSDVIDSQELSKVSREFHSHEFHSHEDMLVVDPKSKEEDKHLKF
ELPKELTPKA-KEESKHSNRIESQENSKLSQEFH-----SLEDKLDLDHK-SEEDKRLKI
:  :  . ** .*..:**: *:*** **:*:***     * ** * :* * .****:**:

RISHELDSASSEVN 314
RISHELDSVSSEVN 278
********.*****
```

2. S100A8: Human (P05109) and bovine (P28782)

```
MLTELEKALNSIIDVYHKYSLIKGNFHAVYRDDLKKLLETECPQYIRKKGADVWFKELD Human
MLTDLECAINSLIDVYHKYSLKKGNYHAVYRDDLKQLLETECPKFMKKKDADTWFKELD Bovine
***:** *:**:********** ***:*********:*******::::** **.******

NTDGAVNFQEFLILVIKMGVAAHKKSHEESHKE 93
NQDGGINFEEFLVLVIKVGLEAHEEIHKE---- 89
* **.:**:***:****:*: **:: *:*
```

3. S100A9: Human (P06702) and bovine (P28783)

```
MTCKMSQLERNIETIINTFHQYSVKLGHPDTLNQGEFKELVRKDLQNFLKKENKNEKVIE Human
MEDKMSQMESSIETIINIFHQYSVRLGHYDTLIQKEFKQLVQKELPNFLKKQKKNEAAIN Bovine
*  ****:* .****** ******:*** *** * ***:**:*:* *****::*** .*:

HIMEDLDTNADKQLSFEEFIMLMARLTWASHEKMHEG-DEGPGHHHKPGLGEGTP-----
EIMEDLDTNVDKQLSFEEFIMLVARLTVASHEEMHNTAPPGQGHRHGPGYGKGGSGSCSG
.********.************:**** ****:**:    * **:* ** *:*

------------------------------------ 114
QGSPDQGSHDLGSHGHGHGHSHGGHGHSHGGHGHSH 156
```

4. C4BPB: Human (P20851) and bovine (Q28066)

```
MFFWCACCLMVAWRVSASDAEHCPELPPVDNSIFVAKEVEGQILGTYVCIKGYHLVGKKT Human
MFFWLMCYLVDVWLISAS---------------------------------------- Bovine
****  * *: .* :***

LFCNASKEWDNTTTECRLGHCPDPVLVNGEFSSSGPVNVSDKITFMCNDHYILKGSNRSQ
----------------DVGHCPDPLLVTDEFSSLEPVNVNDTFMFKCNEHCIFKGSNWSQ
                 :******:**. ****  ****.*.: * **:* *:**** **

CLEDHTWAPPFPICKSRDCDPPGNPVHGYFEGNNFTLGSTISYYCEDRYYLVGVQEQQCV
CRENHTRVTHSPVSKSRDCGPPETPTHGYFEGRDFKSGSTITYYCEARYRLVGTQHQQCI
* *:** .   *:.***** ** .*.******.:*. ****:**** ** ***.*.***:

DGEWSSALPVCKLIQEAPKPE---CEKALLAFQESKNLCEAMENFMQQLKESGMTMEELK
DGEWTSAPPICELIQEAPKPAELELEKAFLAFQESKELCKAIKKFTQRLKKSDLTMEKVK
****:** *:*:********     ***:*******:**:*:::* *:**:* :***::*

YSLELKKAELKAKLL- 252
YSLERKKAKLKAKMLL 198
**** ***:****:*
```

5. TNF: Bovine (Q06599) and ovine (P23383)

```
MSTKSMIRDVELAEEVLSEKAGGPQGSRSCLCLSLFSFLLVAGATTLFCLLHFGVIGPQR Bovine
MSTKSMIRDVELAEEVLSNKAGGPQGSRSCWCLSLFSFLLVAGATTLFCLLHFGVIGPQR Ovine
******************:********** ******************************

EEQSPGGPSINSPLVQTLRSSSQASSNKPVAHVVADINSPGQLRWWDSYANALMANGVKL
EEQSPAGPSFNRPLVQTLRSSSQASNNKPVAHVVANISAPGQLRWGDSYANALMANGVEL
*****.***:* *************.*********:*.:****** ************:*

EDNQLVVPADGLYLIYSQVLFRGQGCPSTPLFLTHTISRIAVSYQTKVNILSAIKSPCHR
KDNQLVVPTDGLYLIYSQVLFRGHGCPSTPLFLTHTISRIAVSYQTKVNILSAIKSPCHR
:*******:**************:************************************

ETPEWAEAKPWYEPIYQGGVFQLEKGDRLSAEINLPDYLDYAESGQVYFGIIAL 234
ETLEGAEAKPWYEPIYQGGVFQLEKGDRLSAEINLPEYLDYAESGQVYFGIIAL 234
** * *******************************:*****************
```

6. OXY: Human (P01178) and ovine (P13389)

```
MAGPSLACCLLGLLALTSACYIQNCPLGGKRAAPDLDVRKCLPCGPGGKGRCFGPNICCA Human
MAGSSLACCLLGLLALTSACYIQNCPLGGKRAVLDLDVRTCLPCGPGGKGRCFGPSICCG Ovine
*** ***************************. *****.***************.***.

EELGCFVGTAEALRCQEENYLPSPCQSGQKACGSGGRCAVLGLCCSPDGCHADPACDAEA
DELGCFVGTAEALRCREENYLPSPCQSGQKPCGSGGRCAAAGICCSPDGCHADPACDPEA
:***************:************** ********. *:************** **


TFSQR 125
AFSQH 125
:***:
```

7. KCNQ1: Human (P51787) and bovine (F1N4R0) (fragment only)

```
MAAASSPPRAERKRWGWGRLPGARRGSAGLAKKCPFSLELAEGGPAGGALYAPIAPGAPG Human
------------------------------------------------------------ Bovine


PAPPASPAAPAAPPVASDLGPRPPVSLDPRVSIYSTRRPVLARTHVQGRVYNFLERPTGW
------------------------------------------------------------

KCFVYHFAVFLIVLVCLIFSVLSTIEQYAALATGTLFWMEIVLVVFFGTEYVVRLWSAGC
-----------------------------------------EIVLVVFFGTEYVVRLWSAGC
                                           ********************

RSKYVGLWGRLRFARKPISIIDLIVVVASMVVLCVGSKGQVFATSAIRGIRFLQILRMLH
RSKYVGIWGRLRFARKPISIIDLIVVVASMVVLCVGSKGQVFATSAIRGIRFLQILRMLH
******:****************************************************

VDRQGGTWRLLGSVVFIHRQELITTLYIGFLGLIFSSYFVYLAEKDAVNESGRVEFGSYA
VDRQGGTWRLLGSVVFIHRQELITTLYIGFLGLIFSSYFVYLAEKDAVNESGQVEFGSYA
*****************************************************:*******

DALWWGVVTVTTIGYGDKVPQTWVGKTIASCFSVFAISFFALPAGILGSGFALKVQQKQR
DALWWGVVTVTTIGYGDKVPQTWVGKTIASCFSVFAISFFALPAGILGSGFALKVQQKQR
************************************************************

QKHFNRQIPAAASLIQTAWRCYAAENPDSSTWKIYIRKAPRSHTLLSPSPKPKKSVVVKK
QKHFNRQIPAAASLIQTAWRCYAAENPDSSTWKIYVRKPSRNHALLSPSPKPKKSAMVKK
************************************:**  *.*:***********.:***

KKFKLDKDNGVTPGEKMLTVPHITCDPPE-ERRLDHFSVDGYDSSVRKSPTLLEVSMPHF
KKFKLDKDNGVSPGEKALVVPHITCDPVAEDRRPEPFSVDGYDSTGEEPPLTVGVGVGVG
***********:**** *.********   :** : ********: .: *  : *.:

MRTNSFAEDLDLEGETLLTPITHISQLREHHRATIKVIRRMQYFVAKKKFQQARKPYDVR
TGPPSALS------------------------------------------------------
*
DVIEQYSQGHLNLMVRIKELQRRLDQSIGKPSLFISVSEKSKDRGSNTIGARLNRVEDKV
------------------------------------------------------------

TQLDQRLALITDMLHQLLSLHGGSTPGSGGPPREGGAHITQPCGSGGSVDPELFLPSNTL
------------------------------------------------------------


PTYEQLTVPRRGPDEGS 676
----------------- 329
```

8. SLC7A11: Human (Q9UPY5) and bovine fragment (B6D7N9)

```
MVRKPVVSTISKGGYLQGNVNGRLPSLGNKEPPGQEKVQLKRKVTLLRGVSIIIGTIIGA Human
------------------------------------------------------------ Bovine

GIFISPKGVLQNTGSVGMSLTIWTVCGVLSLFGALSYAELGTTIKKSGGHYTYILEVFGP
------------------------------------------------------------


LPAFVRVWVELLIIRPAATAVISLAFGRYILEPFFIQCEIPELAIKLITAVGITVVMVLN
------------------------------------------------------------

SMSVSWSARIQIFLTFCKLTAILIIIVPGVMQLIKGQTQNFKDAFSGRDSSITRLPLAFY
------------------------------------------------------------

YGMYAYAGWFYLNFVTEEVENPEKTIPLAICISMAIVTIGYVLTNVAYFTTINAEELLLS
------------------------------------------------------------



NAVAVTFSERLLGNFSLAVPIFVALSCFGSMNGGVFAVSRLFYVASREGHLPEILSMIHV
--------------------FVALSCFGSMNGGVFAVSRLFYVASREGHLPEILSMIHI
************************************:********************.

RKHTPLPAVIVLHPLTMIMLFSGDLDSLLNFLSFARWLFIGLAVAGLIYLRYKCPDMHRP
------------------------------------------------------------

FKVPLFIPALFSFTCLFMVALSLYSDPFSTGIGFVITLTGVPAYYLFIIWDKKPRWFRIM
------------------------------------------------------------

SEKITRTLQIILEVVPEEDKL 501
---------------------- 44
```

9. Colec11: Human (Q9BWP8) and bovine (Q17QH6)

```
MRGNLALVGVLISLAFLSLLPSGHPQPAGDDACSVQILVPGLKGDAGEKGDKGAPGRPGR Human
MKR----ALALMGLAFLCVLRAGAAQQTVDDACSVQILVPGLKGDAGEKGDKGAPGRPGR Bovine
*:     . .*:.****.:* :*  * : ******************************

VGPTGEKGDMGDKGQKGSVGRHGKIGPIGSKGEKGDSGDIGPPGPNGEPGLPCECSQLRK
VGPTGEKGDVGDKGQKGGVGRHGKIGPIGSKGEKGDSGDIGPPGPNGEPGIPCECSQLRK
*********:*******.*********************************:*********

AIGEMDNQVSQLTSELKFIKNAVAGVRETESKIYLLVKEEKRYADAQLSCQGRGGTLSMP
AIGEMDNQVTQLTAELKFIKNAVAGVRETEQKMYLLVKEEKRYLDAQLACQGRGGTLSMP
*********:***:****************.*:********** ****:***********

KDEAANGLMAAYLAQAGLARVFIGINDLEKEGAFVYSDHSPMRTFNKWRSGEPNNAYDEE
KDEAANALLAAYITQAGLARVFIGINDLEREGAFVYADRSPMQTFSKWRSGEPNNAYDEE
******.*:***::***************:******:*:***:**.**************

DCVEMVASGGWNDVACHTTMYFMCEFDKENM 271
DCVELVASGGWNDVACHLTMHFLCEFDKEHV 267
****:************ **:*:******.:
```

10. TNC: Human (P24821) and bovine (A0JN60)

```
MGAMTQLLAGVFLAFLALATEGGVLKKVIRHKRQSGVNATLPEENQPVVFNHVYNIKLPV Human
MGVMTRLWAGLFLALLALPAEGGVLKKVIRHKQQSGMNVTLPEENQPVVFNHVYNIKLPV Bovine
**.**:* **:***:*** :************:***:*.**********************

GSQCSVDLESASGEKDLAPPSEPSESFQEHTVDGENQIVFTHRINIPRRACGCAAAPDVK
GSQCSVDLESASGEKDLAAPSEPRESFQEHTVDGENQIVFTHRINIPRRACGCAAAPDVK
****************** **** ************************************

ELLSRLEELENLVSSLREQCTAGAGCCLQPATGRLDTRPFCSGRGNFSTEGCGCVCEPGW
ELLSRLEELENLVSSLREQCTSGAGCCLQSAEGRVDTRPFCSGRGNFSTEGCGCVCEPGW
**********************:******* * **:************************

KGPNCSEPECPGNCHLRGRCIDGQCICDDGFTGEDCSQLACPSDCNDQGKCVNGVCICFE
KGPNCSEPECPGNCHLHGQCLDGQCVCHEGFTGEDCGQLACPSDCNDQGKCVDGACVCFE
****************:*:*:****:*.:*******.***************:*.*:***

GYAGADCSREICPVPCSEEHGTCVDGLCVCHDGFAGDDCNKPLCLNNCYNRGRCVENECV
GYSGLDCSQETCPVPCSEEHGRCVDGRCVCQEGFAGEDCREPLCLHNCHGRGRCVENECV
**:* ***:* ********** **** ***::****:**.:****.**: **********

CDEGFTGEDCSELICPNDCFDRGRCINGTCYCEEGFTGEDCGKPTCPHACHTQGRCEEGQ
CDEGFTGDDCGELVCPNDCFDRGRCLNGTCSCDEGFTGEDCGQLACPHACHGHGRCDEGQ
*******:**.**:***********:**** *:*********: :****** :***:***

CVCDEGFAGVDCSEKRCPADCHNRGRCVDGRCECDDGFTGADCGELKCPNGCSGHGRCVN
CVCDEGFAGPDCSERRCPSDCHERGRCVDGRCECNDGFTGADCGELQCPRDCSGHGRCVN
********* ****:***:***:***********:***********:**. *********

GQCVCDEGYTGEDCSQLRCPNDCHSRGRCVEGKCVCEQGFKGYDCSDMSCPNDCHQHGRC
GQCVCDEGYTGEDCGQRQCPSDCHGRGRCVDGLCECQPGFQGDDCGEMSCPHDCHQHGRC
**************.* :**.***.*****:* * *: **:* **.:****.********

VNGMCVCDDGYTGEDCRDRQCPRDCSNRGLCVDGQCVCEDGFTGPDCAELSCPNDCHGQG
VNGMCVCDDAYTGEDCRELRCPGDCSQRGRCVDGRCVCEDGFAGPDCADLACPGNCHGRG
*********.*******: :** ***:** ****:*******:*****:*:** :***:*

RCVNGQCVCHEGFMGKDCKEQRCPSDCHGQGRCVDGQCICHEGFTGLDCGQHSCPSDCNN
RCVDGQCVCLEGFTGPDCAQRRCPGDCHGQGRCVDGQCVCHEGFTGPDCAQRSCPNDCSN
***:***** *** * ** ::***.**************:******* **.*:***.**.*

LGQCVSGRCICNEGYSGEDCSEVSPPKDLVVTEVTEETVNLAWDNEMRVTEYLVVYTPTH
WGQCVSGRCVCNEGYTGEDCSQVSPPKDLVVTEVTEETVNLAWDNEMRVTEYLIVYTPTH
 ********:*****:*****:*********************************:******

EGGLEMQFRVPGDQTSTIIQELEPGVEYFIRVFAILENKKSIPVSARVATYLPAPEGLKF
EDGLEMQFRVPGDQTSATIRELEPGVEYFIRVFAILENKKSIPVSARVATYLPTPEGLKF
* ***************: *:*********************************:******

KSIKETSVEVEWDPLDIAFETWEIIFRNMNKEDEGEITKSLRRPETSYRQTGLAPGQEYE
KSIKETSVEVEWDPLDIAFETWEVIFRNMNKDDEEEITKSLRRPETTYRQTGLAPGQEYE
************************:*******:** ***********:**************

ISLHIVKNNTRGPGLKRVTTTRLDAPSQIEVKDVTDTTALITWFKPLAEIDGIELTYGIK
ISLHIVKNNTRGPGLKRVTTTRLDAPSQIEVKDVTDTTALITWSKPLAEIDSIELMYGIK
****************************************** *******.*** ****

DVPGDRTTIDLTEDENQYSIGNLKPDTEYEVSLISRRGDMSSNPAKETFTTGLDAPRNLR
DVPGDRTSIDLTHEENQYSIGNLKPDTEYEVALISRRADMSSNPAKETFTTGLDAPRNLR
*******:****.:*****************:*****.**********************
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RVSQTDNSITLEWRNGKAAIDSYRIKYAPISGGDHAEVDVPKSQQATTKTTLTGLRPGTE
RISQTDNSITLEWRNVKAAADSYRIKYAPISGGDHAEVEVPRSQQTTTRTTLTGLRPGTE
*:************** *** ******************:**:***:**:***********

YGIGVSAVKEDKESNPATINAATELDTPKDLQVSETAETSLTLLWKTPLAKFDRYRLNYS
YGIGVSAVKGDKESDPATINAATELDTPKDLRISDPTETSLTLVWQTPVAKFDRYRLNYS
********* ****:*****************::*: :******:*:**:***********

LPTGQWVGVQLPRNTTSYVLRGLEPGQEYNVLLTAEKGRHKSKPARVKASTEQAPELENL
LPSGQPKEVQLTRDTTSFVLRGLEPGQEYSILLTAEKGRHKSKPARVQASTDHTPELGNL
**:**   *** *:***:**********.:****************:***:::*** **

TVTEVGWDGLRLNWTAADQAYEHFIIQVQEANKVEAARNLTVPGSLRAVDIPGLKAATPY
TVTKAGWDGLRLNWTAADQAYEHFVIQVQEANGVEAAQNLTVPGNLRAVDVPGLKAATPY
***:.******************:******* ****:******.*****:*********

TVSIYGVIQGYRTPVLSAEASTGETPNLGEVVVAEVGWDALKLNWTAPEGAYEYFFIQVQ
RVTIHGVIRGYRTPVLSAEASTGDTPHLGEVTVSEVGWEALKLNWTAPEGVYEQFLIQVQ
 *:*:***:***************:**.****.*:****:***********.** *:****

EADTVEAAQNLTVPGGLRSTDLPGLKAATHYTITIRGVTQDFSTTPLSVEVLTEEVPDMG
EPGKEEAAQNLTVPGGLRSVDLPGLKAATHYSITIRGVTRDFSTAPHSVE----------
*  . **************.***********:*******:****:* ***

NLTVTEVSWDALRLNWTTPDGTYDQFTIQVQEADQVEEAHNLTVPGSLRSMEIPGLRAGT
------------------------------------------------------------

PYTVTLHGEVRGHSTRPLAVEVVTEDLPQLGDLAVSEVGWDGLRLNWTAADNAYEHFVIQ
------------------------------------------------------------

VQEVNKVEAAQNLTLPGSLRAVDIPGLEAATPYRVSIYGVIRGYRTPVLSAEASTAKEPE
------------------------------------------------------------

IGNLNVSDITPESFNLSWMATDGIFETFTIEIIDSNRLLETVEYNISGAERTAHISGLPP
------------------------------------------------------------

STDFIVYLSGLAPSIRTKTISATATTEALPLLENLTISDINPYGFTVSWMASENAFDSFL
------------------------------------------------------------

VTVVDSGKLLDPQEFTLSGTQRKLELRGLITGIGYEVMVSGFTQGHQTKPLRAEIVTEAE
------------------------------------------------------------

PEVDNLLVSDATPDGFRLSWTADEGVFDNFVLKIRDTKKQSEPLEITLLAPERTRDITGL
------------------------------------------------------------

REATEYEIELYGISKGRRSQTVSAIATTAMGSPKEVIFSDITENSATVSWRAPTAQVESF
-------------------------ALTAMGSPKEIIFSDITENAASVSWMAPTTQVESF
                         * ********:********:*:*** ***:*****

RITYVPITGGTPSMVTVDGTKTQTRLVKLIPGVEYLVSIIAMKGFEESEPVSGSFTTALD
RVTYVPIAGGAPSAVTVDGTKTQTRLLRLLPGADYLVSVIALKGFEESEPVSGTLTTALD
*:*****:**:** ************::*:**.:****:**:***********::*****

GPSGLVTANITDSEALARWQPAIATVDSYVISYTGEKVPEITRTVSGNTVEYALTDLEPA
GPSSLVTANITDSEALAMWQPAIAPVDNYVISYTGERVPEITRTVSGNTVEYALTNLEPA
***.************* ****** **.********:******************:****

TEYTLRIFAEKGPQKSSTITAKFTTDLDSPRDLTATEVQSETALLTWRPPRASVTGYLLV
TEYTLRIFAEKGPQKSSTITTKFTTDLDSPRDFTATEVQSETALLTWRPPRASVTGYLLV
********************:***********:**************************

YESVDGTVKEVIVGPDTTSYSLADLSPSTHYTAKIQALNGPLRSNMIQTIFTTIGLLYPF
YESVDGTIKEVVLDPDTTSYSLTDLSPSTYYTARIQALNGTLRSKTVKTIFTTSGVLYRF
*******:***:: ********:******:***:****** ***: ::***** *:** *
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```
PKDCSQAMLNGDTTSGLYTIYLNGDKAEALEVFCDMTSDGGGWIVFLRRKNGRENFYQNW
PRDCSQAMLNGDTTSGVYTIYLNNDKTQKQEVFCDMTSDGGGWIVFLRRKNGREDFYRNW
*:***************:****** **::  ************************:**:**

KAYAAGFGDRREEFWLGLDNLNKITAQGQYELRVDLRDHGETAFAVYDKFSVGDAKTRYK
KAYAAGFGDLKEEFWLGLDTLSKITAQGQYELRVDLRDHGESAHAVYDKFSVGDARTRYR
********* :*********.*.*******************:*.***********:***:

LKVEGYSGTAGDSMAYHNGRSFSTFDKDTDSAITNCALSYKGAFWYRNCHRVNLMGRYGD
LKVEGYSGTAGDSMAYHNGRSFSTFDKDTDSAITNCALSYKGAFWYKNCHRVNLMGRYGD
**********************************************:*************


NNHSQGVNWFHWKGHEHSIQFAEMKLRPSNFRNLEGRRKRA 2201
NSHSQGVNWFHWKGHEHSIQFAEMKLRPSNFRNLEGRRKRA 1746
*.************************************
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Figure A2.1: Amino acid sequence alignments of ovine (or if not available, bovine) with human sequences. Sequences obtained from UniProt (EMBL-EBI) and aligned using Clustal O (1.1.0). Asterisks represent identity, colons represent high similarity, full stops represent low similarity and spaces represent no similarity.

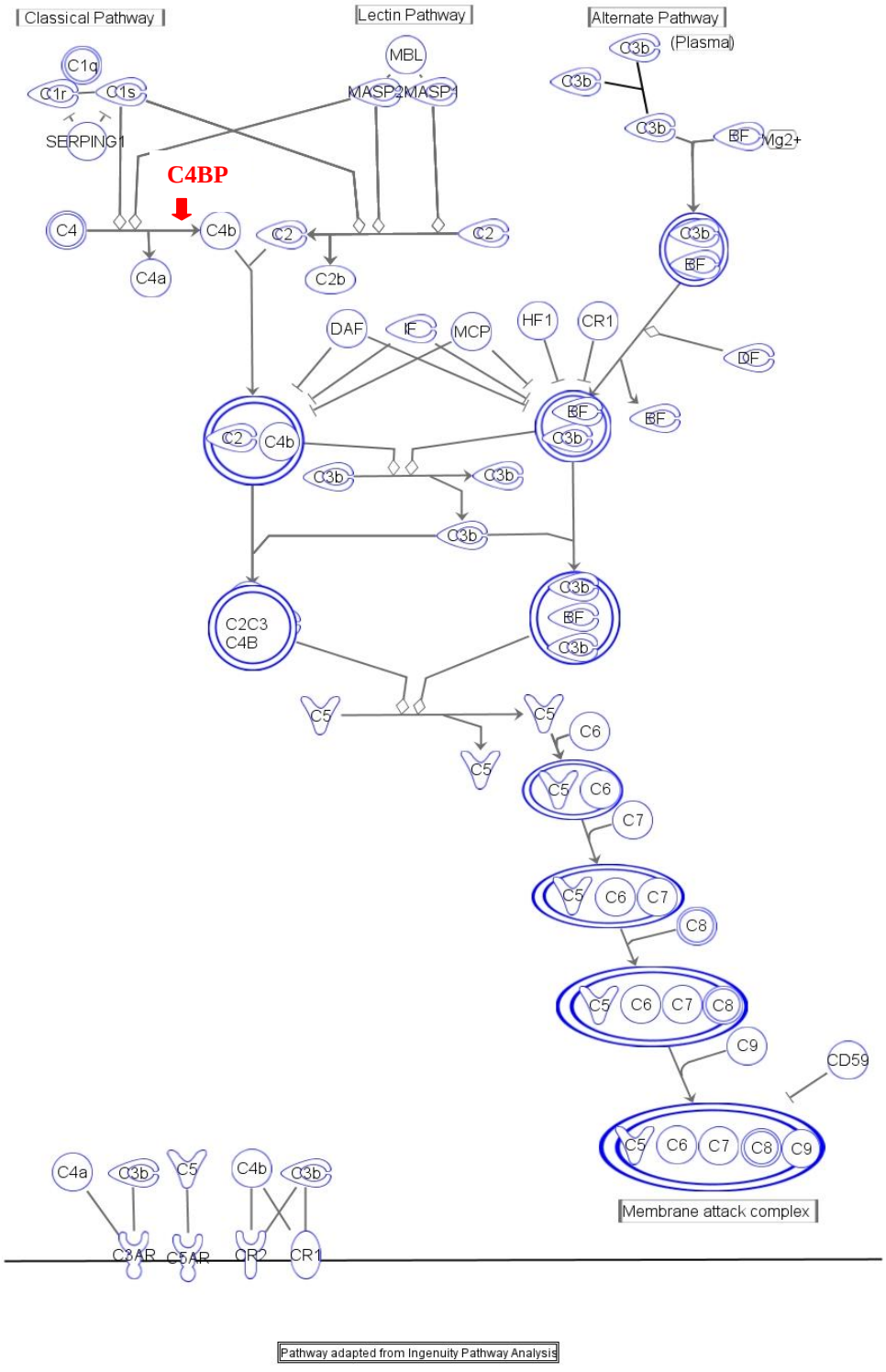

Figure A2.2: The complement cascade illustrating the role of C4BP in the classical and lectin pathways. Figure adapted from Ingenuity Pathway Analysis (IPA).

Table A3.1: Primer names and sequences used in the sequencing and cloning of C4BPB. InFusion primers: red sequence is from the pET22b(+) vector and black from ovine C4BPB sequence.

Primer Name	Primer Sequence 5' to 3'
Degenerate primer forward	TAAGGGCAGCAATYGGAGCC
Degenerate primer reverse	GAAAGGCAAGAARKGCCTTCTC
Gene specific primer 5' RACE	GAAAGGCAAGAAGGGCCTTCTCCAACG
Nested forward RACE primer	TGTCTGCAAAAGCAGAGACTGT
Nested reverse RACE primer	TCTTGGATCAACTCACAGATGG
Gene specific primer 3' RACE	ATGTTTTTTTGGCTTATGTGCCATCTTGTGGAT
InFusion forward primer	TGCGGCCGCACTCGAGTCAGATGTGGGCCACTGTCCT
InFusion reverse primer	GGTGGTGGTGCTCGAGCAACATTTTTGCCTTCAATTTAGCCT
pET SUMO forward primer	TCAGATGTGGGCCACTGTCCT
pET SUMO reverse primer	TCACAACATTTTTGCCTTCAATTTAGCCT

Table A3.2: Optimal assay component concentrations as determined by checkerboard ELISA

Assay component	Function	Optimal conc./dilution
mC4BPB antibody	Coating antibody	1:100
Soya milk	Blocking buffer	1%
Sheep sera infested and control	Antigen	Undiluted
Rabbit anti-sheep C4BPB	Detection antibody	1:100
Mouse anti-rabbit biotinylated	Secondary antibody	1:100
Streptavidin HRP	Conjugate	1:1000

A3.1 General procedures and solutions

Included in this section are all the general procedures and solutions used in chapter 3

A3.1.1 Tris Acetic Acid and EDTA buffer (TAE)

TAE (pH7.4) made up as 50X by dissolving 242g Tris (Sigma) in 500ml H_2O, 100ml 0.5M Na_2EDTA (pH 8.0) and 57.1ml glacial acetic acid. Volume was adjusted to 1L with UP H_2O and stored at room temperature. 1x TAE was made by adding 20ml of the 50X TAE stock solution to 1L UP H_2O

A3.1.2 1% agarose gel

Agarose (Promega) (1g) was added to 100ml 1xTAE (diluted from 50x TAE stock buffers with UP H_2O) and melted in a microwave for 1minute. 10µl Gel Red Nucleic Acid Stain (Biotium) was added.

The gel was loaded using 5µl DNA MW Marker X (Roche) in lane 1 and 3µl of each sample mixed with 1µl Gel Pilot 5x loading dye (Qiagen). Gels were run in a Horizon58 cell (Thistle Scientific) on a ThermoEC (EC105) at 70V for 45 minutes and visualised in a AlphaImager™ 2200 (Alpha Innotech) under UV light.

A3.1.3 LB Agar Plates for JM109 transformed cells

LB agar plates were prepared using a solution of 50ml LB agar (MRI stock), 200µl ampicillin (25mg/ml) (Agilent Technologies), 80µl XGal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) (50mg/ml) (Promega) and 25µl IPTG (Isopropyl β-D-1-thiogalactopyranoside) (1M) (Promega).

A3.1.4 Binding buffer (20mM sodium phosphate, 0.5M NaCl, 20mM imidazole, pH 7.4)

20ml 0.1M sodium phosphate monobasic was diluted with 80ml UP H_2O. 20ml 0.1M sodium phosphate dibasic was diluted with 80ml UP H_2O. 19ml sodium phosphate monobasic was mixed with 81ml sodium phosphate dibasic and the pH checked and altered if necessary to 7.4. Finally, 2.9g NaCl and 0.136g imidazole (Sigma Aldrich) was added and the buffer was stored at 4ºC.

A3.1.5 Binding buffer with 8M urea (for insoluble proteins)

8M urea was added to a stock of binding buffer (as described in section A3.1.4)

A3.1.6 Elution buffers for protein purification column

Elution buffers were based on 20mM sodium phosphate, 0.5M NaCl pH 7.4, therefore the recipe for binding buffer (A3.1.4) was followed until this point. **Elution buffer 1 (50mM):** 0.34g imidazole (Sigma Aldrich) was added to 100ml 20mM

sodium phosphate, 0.5M NaCl. **Elution buffer 2 (200mM):**1.36g imidazole was added to 100ml 20mM sodium phosphate, 0.5M NaCl. **Elution buffer 3 (350mM):** 2.38g imidazole was added to 100ml 20mM sodium phosphate, 0.5M NaCl. **Elution buffer 4 (500mM)**: 3.4g imidazole was added to 100ml 20mM sodium phosphate, 0.5M NaCl.

A3.1.7 Dialysis buffer

200ml 0.1M sodium phosphate monobasic was diluted with 800ml H_2O to make 20mM solution A and 200ml 0.1M sodium phosphate dibasic was diluted with 800ml H_2O to make 20mM solution B. 190ml solution A was added to 810ml solution B and pH adjusted to 7.4. 29g NaCl and 120g urea was added and pH checked and adjusted if necessary.

A3.1.8 Buffers for Protein G column immuno-purification

1. Elution buffer = 0.1M Glycine-HCl (pH 2.7): 7.51g glycine (Fisher Scientific) was dissolved in 800ml UP H_2O and pH adjusted to 2.7 by addition of 1M HCl and solution topped up to 1L by addition of UP H_2O.

2. 1M Tris-HCl pH 9: 121.14g Trizma Base (Sigma Aldrich) was added to 800ml UP H_2O and the pH adjusted to 9 by addition of 1M HCl and solution topped up to 1L by addition of UP H_2O.

A3.1.9 HiTrap Affinity Column Wash Buffers

1. Wash buffer A: 0.5M Ethanolamine, 0.5M NaCl pH 8.3: 3ml ethanolamine was added to 80ml UP H_2O and 4.87g NaCl. The pH was adjusted to 8.3 and the solution topped up to 1L with UP H_2O.

2. Wash buffer B: 0.1M acetate, 0.5M NaCl pH 4: 2.86ml 100% acetic acid was added to 400ml UP H_2O and 14.61g NaCl. The pH was adjusted to 4 and the solution topped up to 500ml with UP H_2O.

A3.1.10 Optimised sandwich ELISA

Buffers: Washing solution = phosphate buffered saline (PBS) (MRI stock) pH7.4 + 0.05% Tween 20 (PBST20); **Carbonate coating buffer** (100 mM) 3.03 g Na2CO3; 6.0 g NaHCO3; 1000 ml distilled water adjusted to pH 9.6; **Blocking buffer** and primary (detection) antibody diluent: 1% soya milk (Infasoy, Cow n Gate) in PBS 0.05% Tween 80 (PBST80).

Antigen = Pooled sera from sheep (n = 12) infested with *P. ovis* in an experimental primary infestation/treatment trial (as described in the Appendix A3.1.11). Pooled week 6 post-infestation sera from 12 animals were used as "infested" sera and pre-bleeds for negative sera controls. A full dilution set of negative sera controls, using the pre-bleed sera from the same trial as the infested sera, were included in each ELISA. Sera concentration used, as determined by checkerboard ELISA, was undiluted.

Sample wells in flat bottomed, high binding 96 well plates (Greiner) were coated in triplicate with 50µl mC4BPB antibody (Novus Biologicals) diluted 1:100 in

carbonate coating buffer, except positive control wells which were coated with 0.12µg rC4BPB diluted in carbonate coating buffer. The plate was covered with a plate sealer and incubated overnight at room temperature, then emptied and washed x 6 by adding 300µl PBS/Tween 20 to each well. This washing was carried out after each of the incubation steps listed below, ensuring the wash buffer was left in the wells for at least 30s at each wash. Following washing, 100µl blocking buffer was added to each well, the plate covered and the ELISA incubated for 1 hr at 37ºC. After washing, 50µl of the undiluted antigen (as described above) was added to each well except the positive controls where PBST80 buffer only was added and the plate incubated for 1 hr at 37°C. Following washing, 50µl of diluted rabbit anti-ovine C4BPB, diluted 1:100 with 1% soya milk, was added to each well except any negative (no primary) controls, where blocking buffer only was added and the plate incubated for 1 hr at 37°C. Finally, 50µl biotinylated secondary antibody raised in mice against rabbit (Abcam) diluted 1:100 with PBST80) was added to each well and the plate incubated for 1 hr at 37°C. Streptavidin HRP Ultrasensitive (Sigma) (50µl) diluted 1:1000 with PBST80, was added to all wells and the plate incubated for 1 hr at 37°C, after which 50µl 3, 3', 5, 5'-Tetramethylbenzidine substrate *solution* (TMB) (KPL) substrate was added to all wells and incubated at room temperature for 10 mins. The reaction was stopped by adding 50µl of TMB Stop solution (100% diethylene glycol, KPL) to each well and the optical density at wavelength 450nm (using 620nm as a reference) was read on a Tecan Sunrise 96-well plate reader.

A3.1.11 Primary and secondary infestation and treatment trial

Twelve sheep scab naïve sheep were pre-bled then infested with 25-50 *P. ovis* mites on the withers area. They were blood sampled weekly for 6 weeks then treated with Dectomax (Pfizer Animal Health) by intra-muscular injection at the recommended dosage rate of 1ml / 33kg bodyweight. Post-treatment, they were blood sampled twice weekly for a month then weekly for further 10 weeks. Lesion size measurements were taken weekly in the primary post-infestation period and for 2 weeks post-treatment. A secondary infestation and treatment trial was started at week 20 and was run as for the primary trial. This trial was named TCT1 throughout the study.

Table A4.1: Mean Hp concentration in sheep serum across a time course of infestation with *P. ovis.* Sera from the TC1 (n = 6) and TC2 (n = 8) trials tested for individual animals in duplicate using a commercial colorimetric assay (ReactivLab).

	TC1		TC2	
Week	**Mean Hp (mg/ml)**	**SE ±**	**Mean Hp (mg/ml)**	**SE ±**
0	0.550	0.191	-	-
1	0.456	0.116	0.077	0.015
2	0.439	0.132	0.120	0.008
3	0.569	0.125	0.173	0.043
4	2.247	0.575	0.090	0.019
5	3.220	0.819	1.122	0.642
6	2.747	0.869	2.170	1.149

Table A4.2: Mean serum Hp concentration in sheep infested with *P. ovis* across a time course of infestation and following successful treatment with an injectable endectocide. Sera from the primary and secondary infestation and treatment trial (TCT1) (n = 12) described in A3.1.11 and tested for individual animals in duplicate by colorimetric assay (ReactivLab). P.i = post-infestation; p.t = post-treatment. *treatment at 6 weeks p.i.

Week p.i/p.t	Mean Hp (mg/ml)	SE ±
0	0.295	0.060
1	0.316	0.099
2	0.673	0.442
3	0.177	0.006
4	0.828	0.414
5	3.327	0.890
***6**	3.533	0.638
6.5	2.986	0.574
7	1.566	0.351
7.5	0.491	0.143
8	0.219	0.042
8.5	0.040	0.042
10	0.000	0.000
15	0.000	0.000
20	0.000	0.000
21	0.435	0.063
21.25	0.978	0.116
22	1.246	0.270
23	0.462	0.078
24	0.577	0.170
25	0.430	0.056
26	0.675	0.164
26.5	0.638	0.198
27	0.433	0.064
27.5	0.342	0.008
28	0.383	0.046
28.5	0.422	0.052

Table A4.3: Mean serum SAA concentration across three time course studies following infestation with *P. ovis.* Sera from the TC1 (n = 6); TC2 (n = 8) and TC3 (n = 6) trials tested for individual animals in duplicate by SAA ELISA (Tridelta Development Ltd). No sample available for TC2 week 0.

	TC1		TC2		TC3	
Week	**SAA (µg/ml)**	**SE ±**	**SAA (µg/ml)**	**SE ±**	**SAA (µg/ml)**	**SE ±**
0	0	0	-	-	0.000	0.000
1	7.340	3.662	0.000	0.000	0.000	0.000
2	9.312	5.071	0.000	0.000	0.000	0.000
3	27.622	7.606	16.354	0.282	24.524	0.394
4	109.034	6.197	40.158	2.817	42.411	0.451
5	185.093	19.156	74.666	3.944	111.569	1.606
6	81.990	16.597	211.291	7.606	190.727	1.690

Table A4.4: Mean serum SAA concentration in sheep infested with *P. ovis* across a time course of infestation and following successful treatment with an injectable endectocide. Sera from the primary and secondary infestation and treatment trial (TCT1) (n = 12) described in A3.1.11 and tested for individual animals in duplicate by SAA ELISA (Tridelta Development Ltd). *treated at week 6 p.i.

Week	Mean SAA (µg/ml)	SE ±
0	0.822	0.528
1	5.166	2.567
2	6.459	5.058
3	3.770	3.312
4	95.244	54.398
5	284.750	71.144
***6**	217.002	46.358
6.5	57.282	34.750
7	19.762	19.762
7.5	0.103	0.092
8	0.000	0.000
9	0.578	0.587
10	0.000	0.000
15	0.000	0.000
20	2.457	2.226
20.2	298.548	79.708
21	49.564	23.954
22	4.512	2.464
23	5.517	2.586
24	52.793	29.336
26	7.311	3.714
26.5	14.214	2.130
27	10.475	1.042
27.5	9.316	0.728

Table A4.5: Mean serum Hp in sheep (n = 12) pre-infestation, at clinical diagnosis as positive for sheep scab and 2 months post-treatment in a field outbreak of sheep scab. Hp measurement by colorimetric assay (ReactivLab); sera tested for individual animals in duplicate.

Time	Hp concentration (mg/ml)	SE ±
Pre-infestation	0.228	0.219
Mid-infestation	0.776	0.382
Post -treatment	0.560	0.168

Table A4.6: Mean serum SAA in sheep (n = 12) pre-infestation, at clinical diagnosis as positive for sheep scab and 2 months post-treatment in a field outbreak of sheep scab. SAA measurement by commercial ELISA (Tridelta Development Ltd); sera assayed for individual animals in duplicate and diluted 1:50.

Time	SAA concentration (µg/ml)	SE ±
Pre-infestation	1.068	0.237
Mid-infestation	10.686	2.659
Post -treatment	3.171	1.166

Table A4.7: Mean normalised OD_{450} ± SE results measuring the ovine serum IgG response to the mite antigen Pso o 2 following infestation with *P. ovis* across a 6-week time course and after treatment with an injectable endectocide (TCT1 trial, n = 12). Sera tested for individual animals in duplicate by Pso o 2 ELISA (MRI) as described in A3.1.10. *treated at 6 weeks p.i.

Week	Mean OD_{450}	SE ±
0	0.573	0.019
1	0.674	0.044
2	1.081	0.126
3	1.197	0.115
4	1.438	0.141
5	1.577	0.138
***6**	2.793	0.062
7	2.760	0.058
8	2.663	0.066
9	1.727	0.098
10	1.580	0.101
11	1.589	0.101
12	1.682	0.175
13	1.542	0.155
15	1.302	0.129
16	0.965	0.095
18	0.853	0.075
20	0.760	0.064
21	1.012	0.060
22	2.378	0.066
23	2.384	0.054
24	2.372	0.076
25	2.403	0.032
27	2.322	0.041
28	2.343	0.064
29	2.293	0.073